# EFFECT OF POLYPHENOL-RICH DARK CHOCOLATE ON ANTHROPOMETRIC, NUTRITIONAL, BIOCHEMICAL AND PHYSIOLOGICAL MARKERS IN NORMAL WEIGHT AND OVERWEIGHT ADULTS

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## Abstract

Polyphenols are phytochemicals widely available in plants. Dark chocolate (DC) is a high source of polyphenols, particularly flavanols. Studies previously reported a beneficial effect of polyphenol-rich dark chocolate (PRDC) on insulin sensitivity and oxidative stress, while its effects on blood pressure, serum lipid levels and inflammation remain unclear. In addition, a research area regarding the effect of PRDC on body weight control emerged recently. Two investigations were carried out. Study I (61 participants) looked at the effect of PRDC on insulin sensitivity in normal weight and overweight adults, while Study II (14 participants) investigated the effect of PRDC on body weight in overweight individuals. Volunteers received 20g of either PRDC (500mg of polyphenols) or placebo DC (low in polyphenols) daily for 4 weeks (Study I) or 12 weeks (Study II). Anthropometric measures and blood, saliva and urine samples were taken. Results showed that 4 weeks of PRDC supplementation decreased insulin levels ($p<0.001$) and HOMA-IR ($p=0.003$), and increased QUICKI ($p<0.001$), but had no significant impact on glucose levels ($p=0.16$). However, participants administered placebo DC showed an increase in insulin ($p=0.014$), HOMA-IR ($p=0.003$), TG ($p= 0.008$), glucose ($p=0.041$) and BMI ($p=0.007$) levels and a decrease in QUICKI ($p=0.013$). No significant changes in blood pressure, other serum lipid levels or glucocorticoid hormones were noted in both groups. In study II, there was an increase in BMI in the whole study population ($p=0.046$) with no significant difference between groups.

Results indicate a potential implication of PRDC in the prevention of risk factors for cardiovascular diseases. Findings also highlight the detrimental effects of placebo DC, and propose the analysis of polyphenol content of different DC brands in the market. The increase in BMI and other markers only in the placebo group after four weeks, suggests that PRDC may counteract the adverse effects of fat and energy in the diet. However, the increase in BMI in both groups after 12 weeks implies further investigations to test the counteracting effect of PRDC over the long term.



Keywords: Polyphenol-rich dark chocolate, placebo DC, insulin sensitivity, HOMA-IR, QUICKI, LDL oxidation, glucose, TG, BMI.

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## List of abbreviations

11βHSD 11-beta hydroxysteroid dehydrogenase
ACE Angiotensin converting enzyme
AgRP Agouti-related protein
AMPK Adenosine monophosphate-activated protein kinase
ANCOVA analysis of covariance
ANOVA Analysis of variance
Apo Apolipoprotein
BAT Brown adipose tissue
BH4 Tetrahydrobiopterin
BIA Bioelectrical impedence
BMI Body mass index
BMR Basic metabolic rate
BP Blood pressure
BSA Bovine serum albumin
CCK Cholecystokinin
CDC Centre for disease control and prevention
CETP Cholesterol ester transfer protein
CI Confidence interval
cGMP Cyclic guanosine monophosphate
CLPr Cocoa liquor procyanidins
COX Cyclooxygenase
CRP C-Reactive protein
CVD Cardiovascular diseases
DBP Diastolic blood pressure
DC Dark chocolate
EDTA Ethylene diamine tetra Acetic acid
ELISA Enzyme-linked immuno-sorbent assay
EC: Epicatechin
eNOS Endothelium nitric oxide synthase
EGCG Epigallocatechin gallate
ERK Extracellular signal-regulated kinase
Fe2+ Ferrous ion
Fe3+ Ferric ion
FFA Free fatty acids
FFQ Food Frequency questionnaire
FRAP Ferric-reducing antioxidant power
G-6-P Glucose-6-phosphate
GAE Gallic acid equivalents
GLP-1 Glucagon-like peptide-1
GLUT Glucose transporter
$H_2O_2$ Hydrogen peroxide
HDL High-density lipoprotein
HOMA-IR Homeostasis-model assessment of insulin resistance
HPA Hypothalamic-adrenal axis
HPLC High performance liquid chromatography
HRP Horseradish peroxidase
HRT Hormone Replacement Therapy
Hs-CRP High sensitivity CRP
IDF International diabetes federation
IGF Insulin growth factor

IL-1 Interleukin 1
IL-6 Interleukin-6
INOS Inducible nitric oxide synthase
IR Insulin resistance
IRS Insulin receptor substrate
LCAT Lecithin-cholesterol acyltransferase
LC-MS Liquid chromatography- tandem mass spectrometry
LDL Low-density lipoprotein
LPL Lipoprotein lipase
MAPK Mitogen-activated protein kinase
MCP-1 Monocyte chemoattractant protein-1
MES-WAT Mesenteric white adipose tissue
MS Metabolic Syndrome
NADPH Nicotinamide adenine dinucleotide phosphate hydrogenase
NaOH Sodium hydroxide
NF-KB Nuclear factor-kappa B
NHANES National Health and Nutrition Examination Survey
NICE National Institute for Health care excellence
NO Nitric oxide
NOS Nitric oxide synthase
NPY Neuropeptide Y
NWO Normal weight obese
ORAC Oxygen radical absorbance capacity
PAF Platelet-Activating factor
PAF-AH Platelet-Activating factor acetyle hydrolase
PAI-1 Plasma Activator inhibitor I
PBS Phosphate buffer saline
PDAD Photo Diode Array Detector
PEPCK Phosphoenolpyruvate carboxykinase
PI3K Phosphatidylinositol3-kinase
PKB Protein kinase B
PPs Polyphenols
PRDC Polyphenol-rich dark chocolate
PYY Peptide YY
QUICKI Quantitative insulin sensitivity check index
RAS Renin Angiotensin system
RCT Randomized controlled trial
RPM Revolutions per minute
SBP Systolic blood pressure
SD Standard deviation
SEM Standard error of mean
SGLT1 Sodium-dependent glucose transporter-1
SCFA Short chain fatty acids
SREBP Sterol regulatory element binding proteins
TC Total cholesterol
TG Triglycerides
TMB Tetramethylbenzidine
TNF-α Tumour necrosis factor alpha
TLR Toll Receptor
UCP1 Uncoupling protein 1
USDA US Department of Agriculture
VAS Visual analogue score
VLDL Very low density lipoproteins

WC Waist circumference
World Health Organization
WHR Waist-to-hip ratio

**Publications**

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# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Obesity, type 2 Diabetes and Cardiovascular diseases: The major prevailing public health challenges

### 1.1.1 Introduction

Obesity, type 2 diabetes and cardiovascular diseases (CVD) currently constitute the major causes of death and disability. These chronic conditions have caused a dramatic increase in health care costs and a decline in the quality of life (Anderson 2004). It is established that a lack of physical activity and a poor nutrition are the major contributors for the considerable increase in the prevalence of obesity and its related complications, with lifestyle modification being the major preventive factor (CDC 2009a). In view of these diseases burden and the diet implication, promoting dietary strategies that aim to improve health are becoming crucial.
Section 1.1 provides a background of the current status of obesity and chronic diseases worldwide, and presents an overview of the pathophysiology of obesity-related complications. This will help in understanding the mechanisms of action of polyphenols, and their potential role in preventing its associated diseases.

### 1.1.2 Overweight and Obesity

#### 1.1.2.1 Definition

Overweight and obesity are terms used to define weight ranges that are considered above to what is regarded as healthy for a given height (CDC 2012). Body mass index (BMI) is the most useful and common tool for the determination of weight status of individuals, and it is adapted to both genders and all ages in adults. BMI provides a rough estimate of body fat percentage for most people, with few exceptions as in the case of athletes (it overestimates fat mass), and older people (it underestimates fat mass) (Snijder et al. 2006). The WHO (World Health Organization) defines a BMI greater than or equal to 30 $Kg/m^2$ as obesity, and a BMI between 25 - 29.9 $Kg/m^2$ as overweight (WHO 2013a). These cut-offs are based on

epidemiological studies which assessed the link between BMI and high mortality risk (PSC et al. 2009).

Waist circumference (WC) is an important tool for assessing adiposity, since it is correlated with total body fat percentage as well as with abdominal fat (Han et al. 1997). WC is considered a surrogate indicator of central fat mass. Central body fat distribution has been regarded as an important contributor to chronic disorders such as CVD and type 2 diabetes (Snijder et al. 2006). WC is considered a better predictor of the risk of diseases than BMI (IDF 2005). Yet, because of the lack of a uniform protocol for the measurement of WC, the latter has not been extensively adopted (Mason and Katzmarzyk 2009), and BMI is still the most widely used tool for assessing weight status. On the other hand, BMI has been shown to be strongly associated with WC (Vazquez et al. 2007). The cut-off points for WC are presented in Table 1.1.

**Table 1.1: Cut-offs for waist circumference in adults**

| | | Waist circumference (cm) |
|---|---|---|
| Healthy or normal: | M | < 94 |
| | F | < 80 |
| Increasing risks: | M | 94–102 |
| | F | 80–88 |
| High risks: | M | > 102 |
| | F | > 88 |

(Adapted from Lean 2002)

*M: Male, F: Female*

*Waist circumference is measured as the midway between the lowest rib and the iliac crest. Classification is based on Scottish Intercollegiate Guidelines Network (1996) and the National Institutes of Health, National Heart, Lung and Blood Institute (1998).*

### 1.1.2.2 Prevalence

The rates of overweight and obesity have dramatically increased over the past two decades, and reached epidemic rates in several regions in the world (WHO 2013a) (View Figure 1.1 on the prevalence of obesity in adults by region). More than one-third (35.7%) of the US adult population is currently obese, with no difference in the

prevalence between men and women (Ogden et al. 2012). The UK seems to have the fastest increasing obesity rate in the developed world, and one of the highest overweight and obesity rates in Europe (Figure 1.2). Around two-thirds of UK men and women are currently either overweight or obese (Diabetes UK 2005). It is estimated that half of the US and UK adult population will be obese by the year 2030 (MRC 2007).

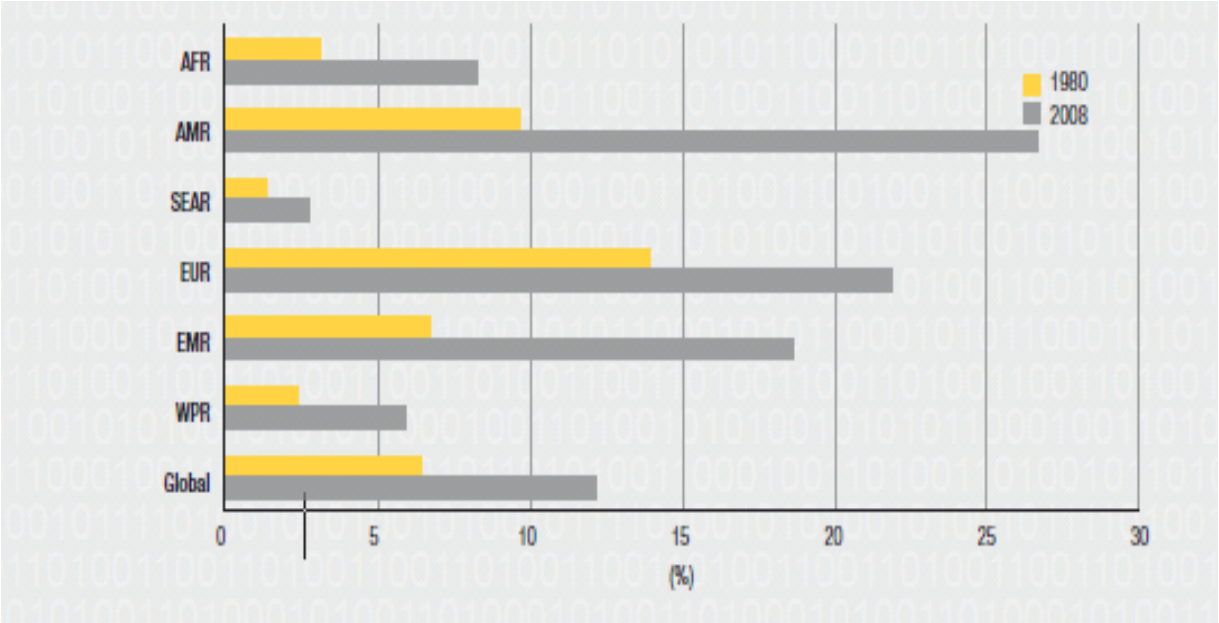



**Figure 1.1: Prevalence of obesity in adults between 1980-2008**

(WHO 2012)

*Prevalence by region. AFR: Africa, AMR: America, SEAR: South-East Asia Region, EUR: Europe, EMR: Eastern Mediterranean Region, WPR: Western Pacific Region.*

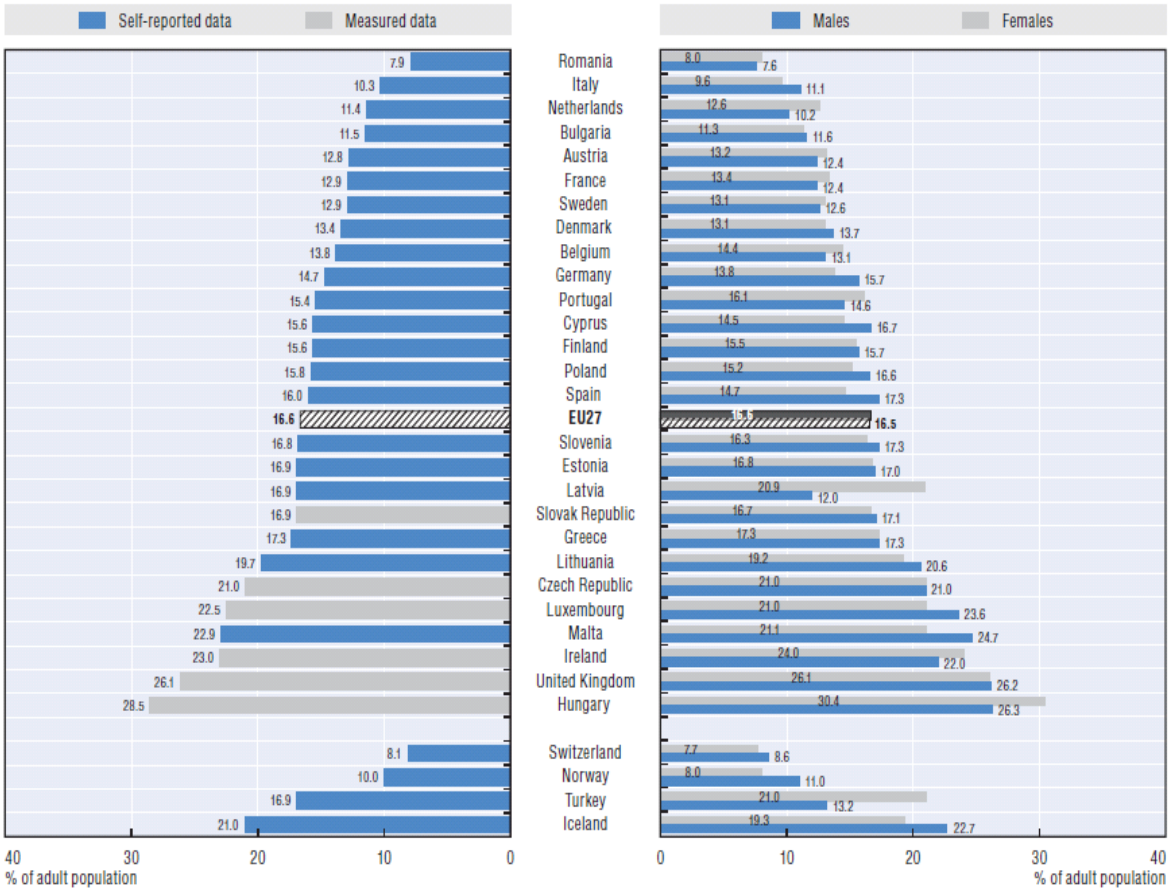



**Figure 1.2: Prevalence of obesity in adults in Europe**
(OECD 2012)
*The prevalence is in 2010 or nearest year*
*EU27: European Union member states (27 countries)*

### 1.1.2.3 The adipose tissue as an active metabolic organ

It is now agreed that the adipose tissue is no longer recognized as a site merely for fat storage (Trayhurn and Beattie 2001; Mariman and Wang 2010), as traditionally known, but an endocrine organ and a secretor of biologically active substances which play a significant role in the occurrence of diseases (Adamczak and Wiecek 2013). In addition to adipocytes, adipose tissue contains macrophages and fibroblasts, which constitute about half of the total number of adipose tissue cells (Hausman 1985).

The adipose tissue secretes inflammatory factors including adipokines (leptin, resistin, adiponectin) and cytokines (like TNF-α (tumor necrosis factor-α) and IL-6 (Interleukin-6)) (Fantuzzi 2005). These factors are implicated in atherosclerosis,

diabetes mellitus, inflammation, endothelial dysfunction and kidney problems (Adamczak and Wiecek 2013). Notably, leptin has been reported to inhibit food intake by halting the appetite stimulator neuropeptide Y (NPY) (Stephens et al. 1995), and to increase energy expenditure (Myers et al. 2008). Leptin rises proportionally to the adipose tissue expansion; however, it becomes dysfunctional in the event of excess fat or obesity. In fact, obese people exhibit higher levels of leptin compared to normal weight people. Yet, this hormone is unable to counter the effects of obesity due to a state called "leptin resistance", caused by many factors such as a defect in the transport of leptin through the blood brain barrier in obese individuals (Myers et al. 2008). Adiponectin has been inversely associated with insulin resistance and inflammation, and its levels decrease with obesity (Kershaw and Flier 2004). As for cytokines, IL-6 levels increase with increasing adiposity (particularly visceral adiposity), and exert a prodiabetic and proatherogenic role by reducing insulin sensitivity, and increasing the coagulation of platelets. Elevated levels of IL-6 have been correlated with low HDL levels and high blood pressure levels (Yudkin et al. 1999). Also, TNF-α, which levels rise in obesity, play a particularly important role in inflammation. TNF-α levels have been shown to increase lipolysis in adipocytes, leading to an increase in circulating free fatty acids (FFA) (Green et al. 2004), and to exert a similar role to IL-6 on insulin sensitivity and platelets (Berry 2008) (The role of FFA in obesity related complications is discussed in section 1.1.4.4). The adipose tissue is also the location of secretion of plasma activator inhibitor-1 (PAI-1), which levels increase in obesity. PAI-1 is upregulated by several factors such as TNF- α, hyperinsulinemia and hypertriglyceridemia, the latter being consequences of obesity and insulin resistance (Lau et al. 2005).

In addition, the white adipose tissue expresses several proteins involved in the Renin Angiotensin system, the latter being responsible for blood pressure homeostasis (View Figure 1.3 on the overview of Renin Angiotensin system). The adipose tissue secretes angiotensinogen (Trayhurn and Beattie 2001), angiotensin I and II, as well as the enzyme ACE (angiotensin converting enzyme). ACE leads to an increase in the antiudiuretic hormone (leading to sodium and water retention), and to an inhibition of the vasodilators bradykinin and kallidin (Shearer et al. 2013). The

expression of angiotensinogen and ACE is higher in visceral adipose tissue compared to subcutaneous adipose tissue (Engeli et al. 2003).

The adipose tissue is also a metabolic regulator involved in glucose haomeostasis (Trayhurn and Beattie 2001), and plays a role in cortisol metabolism due to its implication in the conversion of the hormone cortisone to cortisol through the enzyme 11-hydroxysteroid dehydrogenase type 1 (11 β-HSD 1) existent in this tissue (Chudek et al. 2006).

Lastly, the adipose tissue expresses a variety of hormones and receptors that communicate with the central nervous system, and control numerous biological processes including immune function, energy expenditure and neuroendocrine function. Examples comprise glucagon and insulin receptors, vitamin D receptors and androgen receptors (Kershaw and Flier 2004).

In view of these functions, excess adipose tissue may possibly lead to conditions such as hypertension, hyperglycemia, dyslipidemia, as well as prothrombotic and proinflammatory states (Grundy 2004).

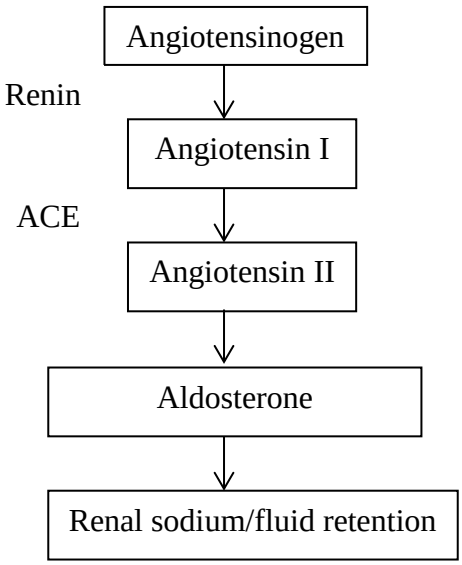



**Figure 1.3: Overview of the Renin Angiotensin system**

(Adapted from Shearer et al. 2013)

*ACE: Angiotensin converting enzyme. The Renin angiotensin system is responsible for blood pressure regulation. Renin converts Angiotensinogen to Angiotensin I. Subsequently, ACE converts Angiotensin I into Angiotensin II (a vasoconstrictor). This stimulates aldosterone release, resulting in an increase in sodium and water retention.*

***The Brown adipose tissue***

In addition to the white adipose tissue, the brown adipose tissue (BAT) has lately received attention for its potential role in obesity and the regulation of energy metabolism. Animal studies have shown that BAT contains a large number of mitochondria, which include a unique protein called uncoupling protein 1 (UCP1). One of the main roles of UCP1 is to dissipate energy in the form of heat. In fact, upon exposure to cold, norepinephrine activates β-adrenergic signalling pathways in BAT, leading to the induction of thermogenesis and the activation of lipolysis. Lipolysis leads to the formation of FFA, the main substrate for the production of heat. FFA stimulate UCP1 which is responsible of uncoupling oxidative phosphorylation and producing energy (Lidell et al. 2014). In addition, thermogenesis leads to the increase in energy expenditure (Virtanen et al. 2009). The mechanism of action of UCP1 involves the peroxisome proliferator-activated receptors (PPARs) (Ali et al. 2014), which are nuclear receptors that act as transcription factors, and are involved in decreasing insulin resistance and dyslipidemia, as well as in attenuating inflammatory response. PPARs consist of PPAR-α, PPAR-β/δ and PPAR-γ, which are expressed in adipose tissue as well as in other organs such as heart and liver (Stienstra et al. 2014). In brown adipose tissue, UCP1 is stimulated by PPAR-γ, the latter being activated by the transcription factor PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1) and involved in mitochondrial biogenesis and oxidative phosphorylation (Lidell et al. 2014). Animal studies have also shown that in addition to the brown adipocytes, some brown-like adipocytes exist within the white adipose tissue. The latter, called inducible brown adipocytes or beige adipocytes have been shown to express UCP1 and PGC-1α genes, and are therefore implicated in thermogenesis (Lidell et al. 2014).

Furthermore, the enzyme AMPK (Adenosine monophosphate-activated protein kinase), which is expressed in BAT but also in white adipose tissue, is thought to play a crucial role in energy metabolism and obesity regulation (Mulligan et al. 2007). This enzyme consists of α, β, and γ subunits (α1, α2, β1, β2, γ1, γ2, γ3), which effects on the function of AMPK remains unclear. In response to fasting and/or exercise, AMPK is stimulated by leptin and adiponectin, and leads to an

increase in fatty acid oxidation in the adipose tissue. AMPK has been suggested to induce thermogenesis in brown adipose tissue via stimulating UCP1 (Ahmadian et al. 2011). AMPK is also expressed in several organs like skeletal muscle and liver, in which functions involve the increase of glycolysis and glucose transport in skeletal muscle, the decrease of gluconeogenesis, the increase in mitochondrial biogenesis, and the decrease in cholesterol synthesis (Daval et al. 2006; Viollet et al. 2007). Therefore, AMPK plays a protective role against obesity and its related complications. AMPK signalling has been reported to be defective in obesity and related diseases (Hardie 2008).

In humans, BAT has been recognized to be abundant in newborns and to decrease with age. It has been suggested that the effect of BAT on energy homeostasis in adults is negligible due to its small proportion (around 700g in a man) (Astrup et al. 1985), and the activity of UCP1 is not physiologically relevant in humans (Cypess et al. 2009). However, in the last few years, it has been reported that adult humans may have significant amounts of BAT which can be implicated in energy balance (Seale and Lazar 2009). An inverse association between BMI and BAT mass has been reported in a study including 1972 patients (Cypess et al. 2009). However, the effect of BAT on adiposity and energy expenditure has been proposed to be temperature-dependent. An exposure to the cold for 2 hours at $19^{o}$C resulted in the activation of BAT mass in 56 volunteers, while this was not the case when the exposure was at higher temperature (27 $^{o}$ C ) (Saito et al. 2000). Also, the increase of BAT volume by 10.5 ± 11.1% ($p<0.001$) and energy expenditure by 5.3 ± 5.9% ($p < 0.001$) has been documented upon an overnight exposure to a temperature of $19^{o}$ C (Chen et al. 2013). Furthermore, exposure to 17°C (2 hours per day) for 6 weeks has been shown to increase the activity of BAT ($p< 0.0001$), and to cause a decrease in body fat (–5.2% ± 1.9%, $P < 0.05$). Yet, no significant changes in BAT and body fat have resulted from an exposure to 27 °C (Yoneshiro et al. 2013). In view of this, inter-individual differences in BAT resulting from differences in cold exposure can be large between humans. These findings, although preliminary, provide a line of evidence that BAT induced via cold temperature can constitute a target for managing obesity and related diseases in humans (Chen et al. 2013). Further studies looking at

the relevance of BAT in humans and its implications in body weight control are then needed.

#### 1.1.2.4 Health Outcomes

Obesity constitutes a major socio-economic challenge due to its impact on the work productivity and the quality of life (MRC 2007), and its burden on the health care system (DH 2013). Unhealthy diets and sedentary lifestyles have notably contributed to the epidemic increase in obesity worldwide (Diabetes UK 2005). Obesity results in multiple consequences including physical (e.g. back pain), social (e.g. isolation), psychological (e.g. low self esteem) and most importantly medical consequences (Ogden et al. 2006; NHS 2011a). The disease risks notably start at the overweight stage and rise with the increase in BMI (NHLBI 2012a). Studies have shown that the prevalence of CVD in the Scottish population is 37% in obese adults (BMI >30 $Kg/m^2$), and 21% in overweight adults (BMI between 25 and 30 $Kg/m^2$), whereas it is around 10% in subjects with BMI<25 $Kg/m^2$. In addition, excess body fat has been linked to an acceleration of atheroma and to an increased risk of thrombosis, stroke and myocardial infarction (Lean 2000). Furthermore, a BMI over 30 $Kg/m^2$ increases by 10 times the risk of type 2 diabetes, whereas a BMI over 35 $Kg/m^2$ increases the risk by 80 times over 10 years (Williams and Pickup 2004). Obesity also raises the risk of death: Morbidly obese individuals have 8-10 years lower life expectancy than normal weight individuals, and it has been stated that each 15 kilograms in excess of normal range raises the risk of premature death by 30 % (OECD 2012). Moreover, obesity has been reported to affect mental health (Chapman et al. 2005), and to interfere with muscle function and strength, possibly leading to impaired physical movement (Hilton et al. 2008) and musculoskeletal disorders (CDC 2013).
On the other hand, it is worth mentioning that normal weight individuals (BMI<25 $Kg/m^2$) might be candidates of a syndrome called normal weight obese (NWO) syndrome, which is characterized by a normal BMI but a high body fat percentage (more than 33.3% in women and more than 23.1% in men) when the latter was assessed by bioelectrical impedance (Romero-Corral et al. 2010).The NWO is clinically important as it is strongly correlated with disease risk. NWO has been shown to raise cardiovascular mortality by 2.2 in women, and to increase

cardiovascular risk factors in men. Increased levels of proinflammatory cytokines like TNF-α have also been noted in individuals with NWO syndrome. Interestingly, there was a positive association between WC and body fat percentage in this study (Romero-Corral et al. 2010).

This highlights one of the drawbacks of BMI in identifying a range of individuals with cardiovascular risk, and emphasizes the need to assess body fat percentage and/or WC in order to recognize individuals at risk, and implement appropriate prevention strategies.

#### 1.1.2.5 Obesity as a "disease"

Despite the fact that the majority of the literature stills consider obesity as a risk factor for cardiovascular diseases rather than a disease by itself (Lean 2000), the serious outcomes of this condition on morbidity and mortality has led to the reconsideration of the definition of obesity. Kopelman (2000) stated that obesity is now perceived as a disease rather than a cosmetic issue. Moreover, in a recent position statement of the American society of endocrinologists, obesity has been recognized as a disease, rather than a result of an adopted lifestyle, as it fulfils the three disease criteria including:

a. Morbidity: mainly due to central obesity which modulates appetite and energy balance, and may lead to insulin resistance, high blood pressure, dyslipidemia, infertility, and non alcoholic fatty liver disease (Mechanick et al. 2012) (The role of central obesity in the occurrence of metabolic syndrome is discussed in section 1.1.4.4). This position is reinforced by the secretory and endocrine properties of adipose tissue, which secretes substances that are linked to the occurrence of diseases (Adamczak and Wiecek 2013), as previously stated.
b. Specific signs and symptoms: Obesity is physically identifiable by the increase in body fat to which manifestations like joint pain and low self esteem are related.
c. Alteration in body functioning: obesity may lead to conditions like osteoarthritis, decrease in body motion and sleep apnea.

Although subject to criticism (due to reasons such as the use of BMI which presents drawbacks in estimating weight status), this new perspective is thought to be beneficial in bringing the attention of health care providers, scientists and the government to the necessity of implementing and improving strategies that aim to prevent and treat obesity, and reduce its financial and social burdens (Mechanick et al. 2012).

### 1.1.3 Atherosclerosis and Cardiovascular diseases

#### 1.1.3.1 Overview

CVD define a cluster of conditions of the heart and blood vessels, including coronary heart disease, peripheral artery disease and stroke. CVD are the leading cause of death in the world (17.3 million people per year). It is expected that by the year 2030, the number will reach 23 million (WHO 2013b). In 2009, CVD were responsible for one third of the deaths in the UK, mainly caused by coronary artery diseases and stroke (BHF 2013; WHO 2013b). CVD are a major consequence of obesity, and constitute a serious burden on the health care system (WHO 2013b).

CVD may be the result of atherosclerosis or "hardening of the arteries", a condition characterized by the accumulation of lipids in the arteries, particularly cholesterol. This leads to the formation of plaque which narrows the artery, and consequently causes ischemia (restriction of blood flow) (Figure 1.4). The rupture of the plaque causes the release of thromobogenic factors in the circulation, and is responsible for blocking blood supply either to the heart, causing myocardial infarction, or to the brain, resulting in stroke (Zebrack and Anderson 2002; NHS 2013).

CVD are viewed as an inflammatory progression because of the involvement of inflammation in the pathogenesis of the atherosclerotic plaque (Shah and Prediman 2009). In fact, due to environmental and/or genetic factors (which cause endothelial injury), there is a release of the nuclear factor-kappa B (NF-κB) (a transcription factor expressed in many cell types including adipose tissue, liver and central nervous system, and a chief regulator of inflammation). NF-κB activates chemokines

(small cytokines) and adhesion molecules (Baker et al. 2011). Chemokines such as MCP-1 (monocyte chemoattractant protein-1) attract monocytes from the circulation, and adhesion molecules allow the monocytes to cross the endothelial wall to be converted to macrophages, constituting a key step in atherosclerosis (Zebrack and Anderson 2002). Macrophages are then responsible for the secretion of cytokines (TNF-α, IL-6 and IL-1) and metalloproteinases (have a role in the migration of vascular smooth muscle cells (Lee et al. 2008)) in the vessel walls, leading to the initiation of the inflammatory cascade. LDL (Low density lipoproteins) cholesterol, which have been oxidised in the artery walls, are then taken by macrophages. This results in the formation of foam cells and eventually, the atherosclerotic plaque. At this stage, some inflammatory factors help in plaque growth and destabilization, and might result in its rupture (Zebrack and Anderson 2002). Furthermore, proinflammatory enzymes like NADPH (nicotinamide-adenine dinucleotide phosphate) oxidase and xanthine oxidase are implicated in endothelial dysfunction (Maytin et al. 1999). NADPH oxidase leads to vascular dysfunction through the formation of reactive oxygen species (ROS) (Steffen et al. 2006). Xanthine oxidase binds to endothelial cells, and generates oxygen and hydrogen peroxide ($H_2O_2$) species when reacting with xanthine or hypoxanthine (White et al. 1996).

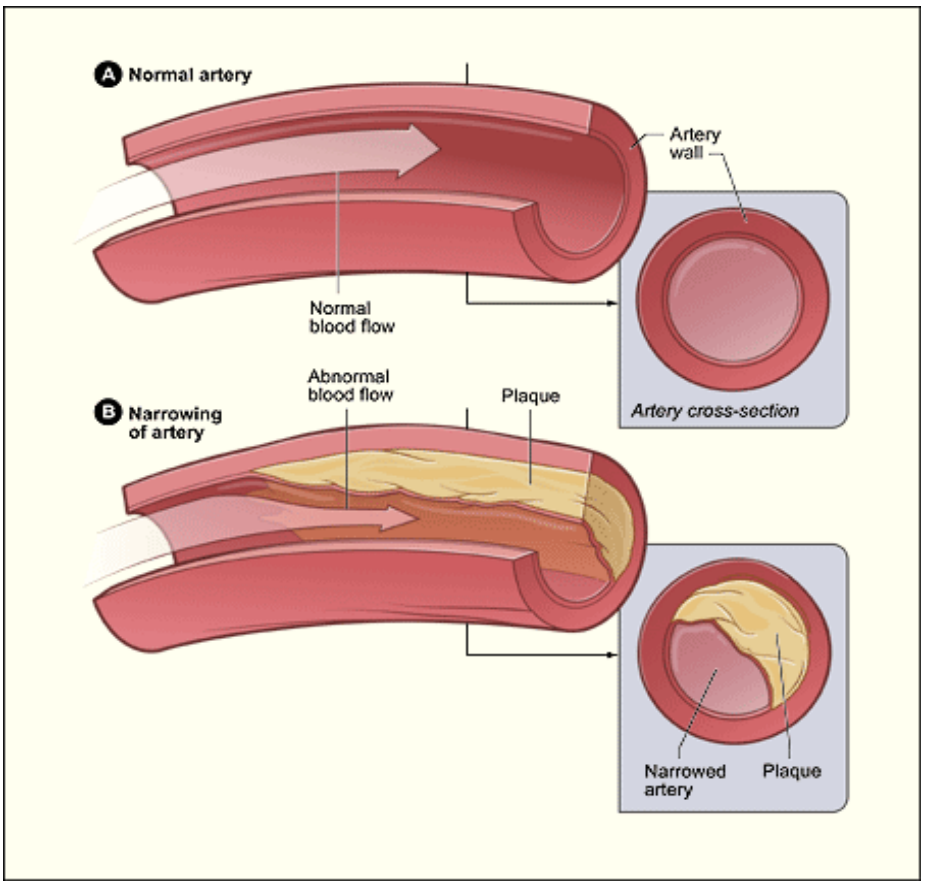



**Figure 1.4: Development of plaque in atherosclerosis**

(NHLBI 2011)
*The accumulation of LDL particles and their oxidation in the artery walls result in their scavenging by macrophages and the formation of foam cells. This leads to the formation of atherosclerotic plaque which restricts normal blood flow.*

### 1.1.3.2 Nitric oxide, endothelial dysfunction and oxidative stress

Nitric oxide (NO) is a biologically active free radical implicated in several reactions in the body, and is the most important endothelium-derived vasodilator (eNOS) (Dobutovic et al. 2011). NO is responsible for the proliferation of smooth muscle cells, as well as the inhibition of platelet aggregation and thrombosis. NO has been also involved in the haemostasis of blood pressure through mechanisms involving vasodilatation (Napoli and Ignarro 2009).

NO is produced by a series of NO synthase (NOS) enzymes, the main ones being: neuronal NOS, endothelial NOS and inducible NOS (iNOS) (Liu and Hang 2008). The mechanisms of action of NOS enzymes involve catalysing the oxidation of

L-arginine to NO and citrulline (Stuehr et al. 2004). NO then binds to guanylyl cyclase and results in increased levels of cGMP (cyclic guanosine monophosphate), resulting in the increase in vasodilatation, the relaxation of smooth muscle cells (Napoli and Ignarro 2001; Napoli and Ignarro 2006) and the decrease in platelet aggregation (Jerca et al. 2002) (Figure 1.5).

Endothelial dysfunction refers to a state during which the endothelium loses its vasodilatory and anti-aggregation properties (Avogaro et al. 2011), leading to an increase in the risk of thrombosis, diabetes mellitus, hypertension and cardiovascular and cerebrovascular diseases (Napoli and Ignarro 2001). The reduction in NO levels has been suggested to be responsible for the development of vascular problems; however, it is not clear yet whether this reduction is a cause or a consequence of endothelial dysfunction. Nevertheless, it is known that synthesis and bioavailability of NO are crucial for maintaining a healthy endothelium. Decreased levels of NO could be due to several factors such as a decrease in NOS enzyme levels, or a decrease in the bioavailability of NO (Napoli and Ignarro 2009). Studies have shown that mice lacking certain forms of NOS are hypertensive, have endothelial dysfunction, and demonstrated a more severe response to a high cholesterol diet-induced atherogenesis. This can also be applicable to humans, since the similarity in the isoforms of NOS between mice and humans ranges between 80-96% (Liu and Huang 2008). It has also been stated that some risk factors for coronary heart diseases like hypertension, diabetes and hypercholesterolemia can reduce the levels of L-arginine, leading to a reduction in NO bioavailability **(**Böger and Ron 2005).

Oxidative stress, a contributor to endothelial dysfunction, is described as an imbalance between pro-oxidants (ROS) and antioxidants (supposed to repair the damage), resulting in an alteration of the redox signalling (Jones 2006). Oxidative stress is of particular importance, as it may contribute to several chronic diseases such as CVD, cancer and hypertension (Urquiaga and Leighton 2000). Oxidative stress results in the formation of ROS, which act as scavengers for NO, and cause endothelial dysfunction (Steffen et al. 2006). In particular, oxidised LDL can reduce the uptake of L-arginine by uncoupling NOS enzymes, giving rise to ROS (Napoli

and Ignarro 2009). Moreover, in the presence of oxidants like superoxide (which can originate through several pathways like xanthine oxidase, NADPH oxidase, and endothelial NOS), NO gives peroxynitrite (an oxidant implicated in cellular dysfunction). Peroxynitrite reduces the bioavailability of NO and leads to an increase in inflammation and oxidative damage (Radi 2004). Consequently, atherosclerosis may be caused by the disruption of the balance between the bioactivity of NO and oxidative stress (Napoli and Ignarro 2001).

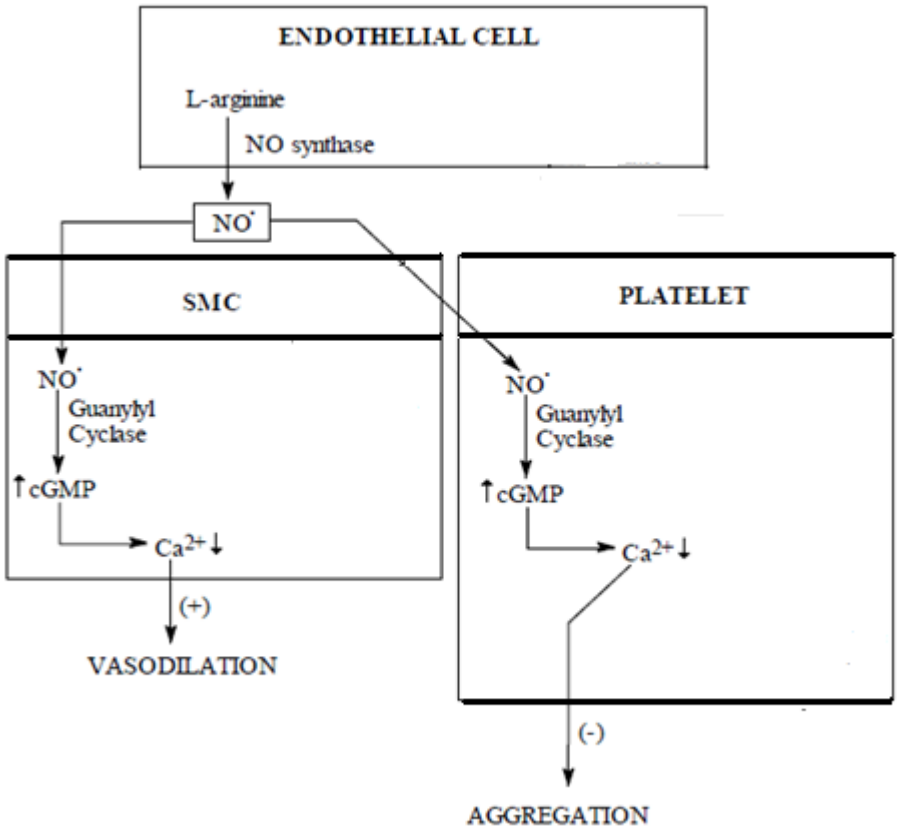



**Figure 1.5: Synthesis and mechanism of action of nitric oxide**

(Adapted from Jerca et al. 2002)
*SMC: Smooth muscle cells, cGMP: Cyclic guanosine monophosphate, NO: Nitric oxide NO synthase catalyses the oxidation of L-arginine to NO. NO results in the increase in cGMP levels leading to vasodilatation of smooth muscle cells, and to inhibition of platelet aggregation.*

#### 1.1.3.3 Risk Factors

##### *a. High blood pressure or Hypertension*

Hypertension is known as the "silent killer" (NHS 2012a), as it might be undetected for years until a cardiovascular event like a stroke or a heart attack occurs (Cataldo 2002). It is estimated that around one in 3 adults in the US is hypertensive (NHLBI 2012b). However, approximately one third of these individuals are undiagnosed (Cataldo 2002).

A high blood pressure is defined as an elevated systolic blood pressure (SBP) and/or diastolic blood pressure (DBP) above the normal levels (NHLBI 2012b) (view Table 1.2 on the classification of blood pressure in adults). Prehypertension should be carefully considered since it can contribute to hypertension, and to an increased risk of heart diseases. In fact, the Framingham Heart study showed that men with prehypertension have 3.5 times more risk of having heart attacks than normotensive men (Qureshi et al. 2005). Hypertension leads to or worsens atherosclerosis by damaging the walls of the arteries, and increasing the risk of developing plaques and clots. This results in the occurrence of stroke and other heart conditions (Cataldo 2002). Hypertension is responsible for 37% of deaths in the western population (Martiniuk et al. 2007), and for 54% of strokes and 47% of ischemic heart diseases globally (Lawes et al. 2008). The risk rises proportionally to blood pressure (Lewington et al. 2002).

##### *b. Dyslipidemia and oxidised LDL*

Dyslipidemia is a disorder of the metabolism of lipoproteins, expressed by high total serum cholesterol, high LDL cholesterol (the small and dense LDL are considered the most atherogenic), low HDL cholesterol and/or elevated triglycerides (TG) levels (Toth 2008). Dyslipidemia is considered an increasing problem and one of the major risk factors for CVD. The condition may be caused by many factors, such as an unhealthy diet (high in trans-fatty acids, saturated fatty acids and cholesterol and low in fiber), low levels of physical activity, overweight and obesity (mainly abdominal obesity), smoking and high alcohol consumption. High TG and low HDL levels play

an important role in the development of atherosclerosis (Talayero and Marks 2011), and are implicated in atherogenic dyslipidemia, the latter being described as a triad of high levels of TG and small and dense LDL, and low levels of HDL.

LDL oxidation is one of the most important factors in the development of atherosclerosis, and results from the oxidation of LDL particles in the artery walls leading to these particles being scavenged by the monocytes, and resulting in the formation of fibrous plaque (Sorace et al. 2006), as previously discussed. Oxidised LDL is more crucial in atherosclerosis than LDL, and is a main contributor to endothelial dysfunction and to an increase in arterial thickness (Li and Mehta 2000). Oxidised LDL exerts its pro-oxidant and pro-inflammatory effects by stimulating the release of factors involved in the pathogenesis of atherosclerosis such as cytokines (TNF-α and IL-1), and chemokines like interleukin-8 (a chemokine with angiogenic properties) (Claise et al. 1996). Higher levels of oxidised LDL have been reported in subjects with hyperlipidemia compared to normolipemic subjects (Liu et al. 2004). Oxidised LDL has been suggested to be inversely correlated to HDL levels, as an increase in the latter cause an inhibition of LDL oxidation (Holvoet et al. 1998). The mechanisms involve a role of HDL in reversing cholesterol transport through the lecithin-cholesterol acyltransferase (LCAT) enzyme, which is activated by Apo A-1 (apolipoprotein A-1), the major protein of HDL. This results in the transfer of cholesterol from peripheral tissues to the liver (Itabe et al. 1999). Also, HDL has been shown to inhibit the stimulatory effect of LDL oxidation on monocyte infiltration. This happens through the action of HDL associated enzymes, such as paraoxonase which inhibits the oxidation of PAF-AH (Platelet-activating factor acetyl hydrolase) enzyme. The latter enzyme suppresses PAF (Platelet-activating factor) which contributes to atherosclerosis by stimulating the formation of superoxide anions by macrophages. PAF-AH has been reported to increase the degradation of oxidised lipids in the artey walls. PAF-AH is secreted by macrophages and platelets, and circulates in association with HDL. Therefore, HDL has been suggested as an antagonist to oxidised LDL in the event of CVD risk (Mertens and Holvoet 2001). However, a weak or no correlation between LDL oxidation and plasma LDL cholesterol has been noted (Holvoet et al. 1998; Baba et al. 2007b).

*c. Other risk factors*

Factors such as overweight and obesity, smoking, high alcohol consumption, family history of premature cardiovascular diseases (defined by the occurrence of CVD at an age less than 55 years in males and 65 years in females) and age (men≥45 years, women ≥55 years) may all contribute to an increase in cardiovascular risk (Lee and Nieman 2010; WHO 2013b).

**Table 1.2: Classification of blood pressure in adults**

| Blood Pressure classification | SBP (mmHg) | DBP (mmHg) |
|---|---|---|
| Normal | < 140 | and <90 |
| Stage 1 Hypertension | ≥140 | or ≥ 90 |
| Stage 2 Hypertension | ≥ 160 | or ≥ 100 |
| Severe Hypertension | ≥ 180 | or ≥ 110 |

(Adapted from the National Institute for Health and Care Excellence (NICE) 2011)
*SBP: Systolic blood pressure, DBP: Diastolic blood pressure.*

### 1.1.4 Type 2 Diabetes and Insulin Resistance

#### 1.1.4.1 Definitions

Type 2 diabetes is a globally common complex disease characterized by insulin resistance (IR) and a gradual destruction of pancreatic beta cells, and ultimately leading to an increase in glucose levels (Deckert et al. 1992) (Figure 1.6). IR is the state when the body is producing insulin but unable to use it effectively. IR is a leading factor for the occurrence of type 2 diabetes (NIDDK 2013), and may precede the development of the disease by more than 10 years (Zimmet et al. 1992).

#### 1.1.4.2 Prevalence

Type 2 Diabetes is now considered one of the biggest health challenges worldwide. In the UK, the prevalence of diabetics has dramatically increased over the past 2

decades, and reached more than 2.6 million. It is also suggested that the undiagnosed cases account for an additional half a million of diabetics. It is estimated that by the year 2025, the number will rise to four millions. In the US, type 2 diabetes has reached epidemic levels, and around 26 million individuals currently suffer from type 2 diabetes (NDEP 2013). Type 2 diabetes is mainly associated with obesity (Diabetes UK 2012), and its diagnosis at an early stage is extremely important, as 50% of the cases of type 2 diabetes can be avoided if prevention strategies are implemented (Diabetes UK 2012). Type 2 diabetes is a major risk factor for CVD (Bartels et al. 2007). CVD and type 2 diabetes account together for 75% of the deaths worldwide (WHO 2003).

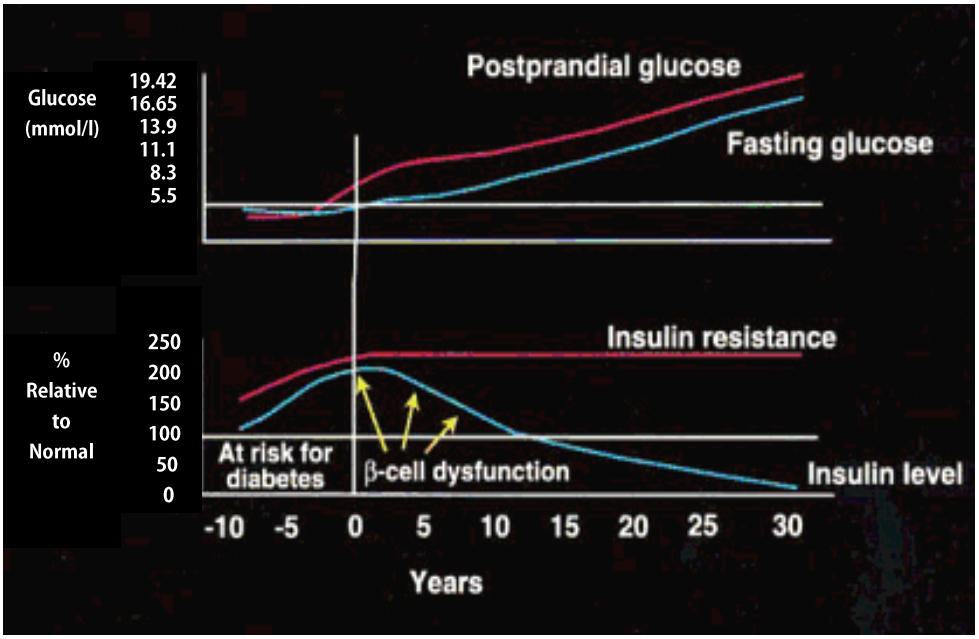



**Figure 1.6: Progression of type 2 diabetes**

(Adapted from Bergenstal and Bailey 2000)
*Insulin resistance causes an increase in insulin secretion and a gradual destruction of β-cells. This eventually leads to a decrease in insulin secretion and an increase in glucose levels.*

### 1.1.4.3 Risk factors

Diabetes type 2 is considered to be the result of an interaction between genetic and environmental factors (Diabetes UK 2005). Overweight and obesity, particularly

central obesity, remain the most important contributors for type 2 diabetes. IR is very common in overweight individuals, as 80% of individuals diagnosed with type 2 diabetes are overweight (Astrup and Finer 2000). Ethnicity and age may also exert an influence. Age increases the risk, and some ethnic groups like Hispanic have a higher risk of type 2 diabetes than others (Oldroyd et al. 2005).

#### 1.1.4.4 The metabolic Syndrome

The metabolic syndrome (MS) is defined as a clustering of risk factors for type 2 diabetes and CVD, resulting from obesity (particularly visceral obesity) and IR. MS consists of several risk factors such as abdominal obesity, dyslipidemia, high blood pressure and glucose intolerance. The National cholesterol education program adult treatment panel III defines MS as the presence of 3 of the five following criteria: high waist circumference (≥102 cm in men and ≥ 88cm in women), high TG levels (≥ 1.7 mmol/l), low HDL levels (< 1 mmol/l in men and < 1.3 mmol/l in women), elevated fasting glucose levels (≥ 5.5 mmol/l) and high blood pressure (≥ 135/80 mmHg or hypertension medication). Knowing that there has been some confusion over the purpose and definition of this syndrome (Blaha and Elasy 2006), the main purpose of discussing MS is to demonstrate how obesity and its resultant IR might lead to a series of abnormalities, and subsequently to the development of chronic diseases.

One approach considers obesity, particularly visceral obesity, an essential contributor to the syndrome, which can lead to IR and consequently to an increased risk of type 2 diabetes, CVD and other diseases (Grundy et al. 2004) (Figure 1.7). Another approach considers IR as located in the heart of pathophysiological mechanisms that originate this syndrome, and that obesity is thought to exacerbate IR, but is not an essential contributor to its occurrence (Reaven 1988). The latter approach is, in fact, the theory of the founder of this syndrome. IR results in conditions like type 2 diabetes, CVD, polycystic ovarian syndrome and nonalcoholic fatty liver disease (Reaven 1988). However, it has been subsequently widely proven that obesity is strongly associated with the development of insulin resistance (Kahn et al. 2006; Qatanani and Lazar 2007), rendering body fat a crucial factor in the occurrence of MS.

FFAs play a central role in the pathophysiology of MS and in the occurrence of type 2 diabetes (Reaven et al. 1988). The increase in body fat, particularly visceral fat, causes an increase in FFA in the circulation, and results in a set of pathological consequences (Snijder et al. 2006). The pathophysiology of the metabolic syndrome and the role of obesity are illustrated in Figure 1.8.

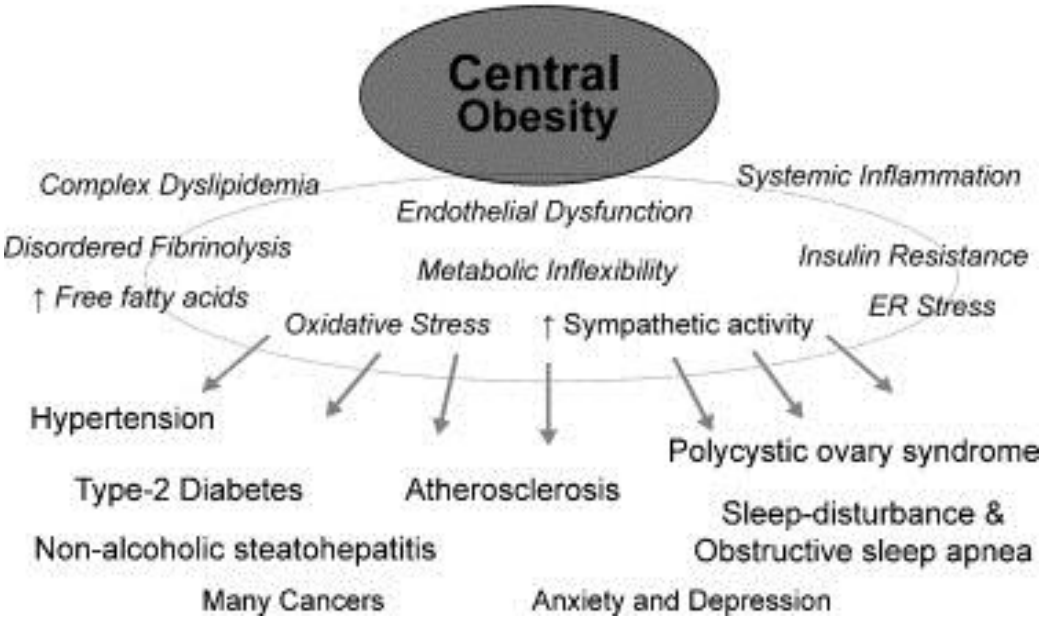



**Figure 1.7: Consequences of central obesity and its implications in the metabolic syndrome**

(Adapted from Dixon 2010)
*ER: Endoplasmic reticulum*

*a. Obesity and insulin and glucose metabolism*

The increase of FFA in the circulation, mainly due to abdominal obesity, leads to a decrease in the uptake and oxidation of glucose by muscles and other organs. The pancreas will then offset the decline in the uptake of glucose by increasing insulin secretion, resulting in hyperinsulinemia (Jensen et al. 2006; Snijder et al. 2006), and eventually leading to IR. IR is due to many factors such as the downregulation of insulin receptors caused by chronic hyperinsulinemia (Gavin et al. 1974**).** In addition, FFA accumulate in some organs (mainly liver and muscle but also pancreas), and cause lipotoxicity or "ectopic fat storage" (Kahn and Flier 2000). This will affect the functioning of pancreatic β cells. In the liver, the excess of FFA

causes an increase in gluconeogenesis and a decrease in the clearance of insulin from the liver (caused by the inhibition of the binding of insulin molecules to their receptors due to excess FFA). This contributes to or worsens hyperinsulinemia (Svedberg et al. 1992). Furthermore, the adipose tissue secretions (section 1.1.2.3) play a pivotal role in the manifestation of MS (Hermans and Amoussou-Guenou 2005).

*b. Obesity, insulin resistance and dyslipidemia*

Insulin plays a significant role in the metabolism of lipids. Dyslipidemia is recognized as a common feature in IR (Lamarche and Mauger 2005). Under normal conditions, insulin inhibits gluconeogenesis and lipolysis, induces lipogenesis (Saltiel and Khan 2001), and increases the activity of LPL (lipoprotein lipase) in the postprandial state (resulting in the uptake of fat by the adipose tissue) (Panarotto et al. 2002). However, in the event of IR, there is an abnormality in lipid metabolism: the insulin-mediated suppression of lipolysis and the stimulation of lipogenesis are defective. The reasons are mainly due to the fact that insulin resistant subjects have a decrease in insulin-stimulated glucose transporter (precursor of TG synthesis) (Kahn and Flier 2000), which affects the rate-limiting step in the synthesis of Triglycerides (Farese et al. 2000). Moreover, a problem in the regulation of LPL has been noted in obese and insulin resistant individuals (Yost et al. 1995). This results in the increase in the accumulation of FFA levels (Kahn and Flier 2000).

In the liver, the excess of FFA resulting from obesity is secreted in form of VLDL (Very low density lipoproteins) particles rich in TG and Apo B 100 (the protein transporter of VLDL and LDL) (Bjorntorp 1990), resulting in hypertriglyceridemia, and in most of the lipid abnormalities associated with MS. Also, collisions between VLDL and HDL particles through to the cholesteryl-ester transfer protein (CETP) enzyme lead to a transfer of TG to HDL, in exchange of cholesteryl esters. HDL molecules rich in TG become substrate for the hepatic lipase and LPL, which hydrolyse their TG. Reduced HDL molecules partially lose their cortex, and are then eliminated by the kidneys. As a result, smaller and denser HDL (HDL3) will be abundant at the expense of larger, less dense and more cardioprotective particles (HDL2). Furthermore, LDL particles enriched in TG become substrates for the hepatic lipase and LPL, which hydrolyse their TG. Thus, these LDL particles

become small and dense. The metabolic syndrome is thus characterized by the presence of small and dense LDL, high TG and low HDL levels, albeit serum LDL levels are not necessarily increased (Goff et al. 2005; Hermans and Amoussou-Guenou 2005).

*c. Obesity, insulin resistance and hypertension*

It has been acknowledged that obesity is responsible for 65-75% of essential hypertension (Hall et al. 2001). Although not clearly elucidated, proposed mechanisms involve a high salt intake accompanying a high calorie diet (Carroll et al. 1997), and an increase in renal sodium tubular absorption. This increase in sodium reabsorption has been explained by the activation of the renin-angiotensin system (Atlas 2007) and the increase in renal sympathetic activity; the latter has been documented to be elevated in obese adults (Esler 2000). Indeed, a high blood pressure has been noticed in dogs fed a high fat diet, who exhibit hyperinsulinemia, sodium retention and hypertension similarly to obese humans (Hall et al. 1993). Furthermore, under normal conditions, insulin is known to exert a vasodilatory effect on the kidneys, and this effect is mediated by nitric oxide (Hayashi et al. 1997). However, in the case of IR, the insulin-dependent vasodilatory role is impaired due to a defect in insulin signalling mechanisms (Li et al. 2010), resulting in an increase in hypertension risk.

Overall, inflammation and alterations in FFA metabolism play a pivotal role in the occurrence of MS. Excess FFA released from the adipose tissue can increase hepatic glucose production and suppress the ability of insulin to increase cellular glucose uptake, leading to IR (Snijder et al. 2006). Furthermore, oxidised LDL (which levels increase with the increase in adipose tissue) can contribute to IR by impairing insulin signalling, decreasing adiponectin and causing pancreatic β-cell death. Oxidised LDL is thought to be an initiator of the MS (Holveot et al. 2008). IR can be also induced or exacerbated by inflammatory cytokines secreted by the adipose tissue. In fact, the increase of lipid deposition in adipose tissue can activate NF-κB which stimulates intracellular pathways that cause IR. However, the mechanisms involved in the effect of FFA on NF-κB remain unclear. Furthermore, TNF-α decreases

insulin signalling (Shoelson et al. 2006). IL-6 can stimulate inflammation directly or by inducing the secretion of CRP in the liver (Park et al. 2004), and can contribute to IR. Moreover, MCP-1, which is highly expressed in visceral adipose tissue of obese individuals, participates in the recruitment of monocytes into the arterial wall and in the activation of macrophages in the atherosclerotic plaque. This will result in oxidised LDL being incorporated into macrophages. IL-8 plays a similar role to MCP-1 and attracts monocytes into the arterial wall (Trøseid et al. 2004). MCP-1 has been shown to induce IR in rats (Panee 2012). Oxidised LDL has also been involved in increasing the infiltration of monocytes (Holveot et al. 2008). As for lipid levels, the excess of FFA can cause dyslipidemia by increasing VLDL production and TG levels, and decreasing HDL levels (Hermans and Amoussou-Cuenou 2005). Oxidised LDL stimulates LPL which leads to an increase inTG levels (Holveot et al. 2008). IR can also contribute to dyslipidemia due to impairment in insulin-mediated suppression of lipolysis, which increases FFA release from adipose tissue (Kahn and Flier 2000). In addition, IR can lead to increased blood pressure due to the impaired role of insulin as a vasodilator (Hayashi et al. 1997). Inflammation can be an additional risk factor for hypertension due to the inhibitory effect of CRP on NO production (Ghanem and Movahed 2007) (Figure 1.8).

In view of the inter –related abnormalities, there is a need for effective intervention strategies that target different components of the MS including inflammation, oxidative stress, IR, dyslipidemia, blood pressure and weight management in order to reduce the incidence of type 2 diabetes and CVD.

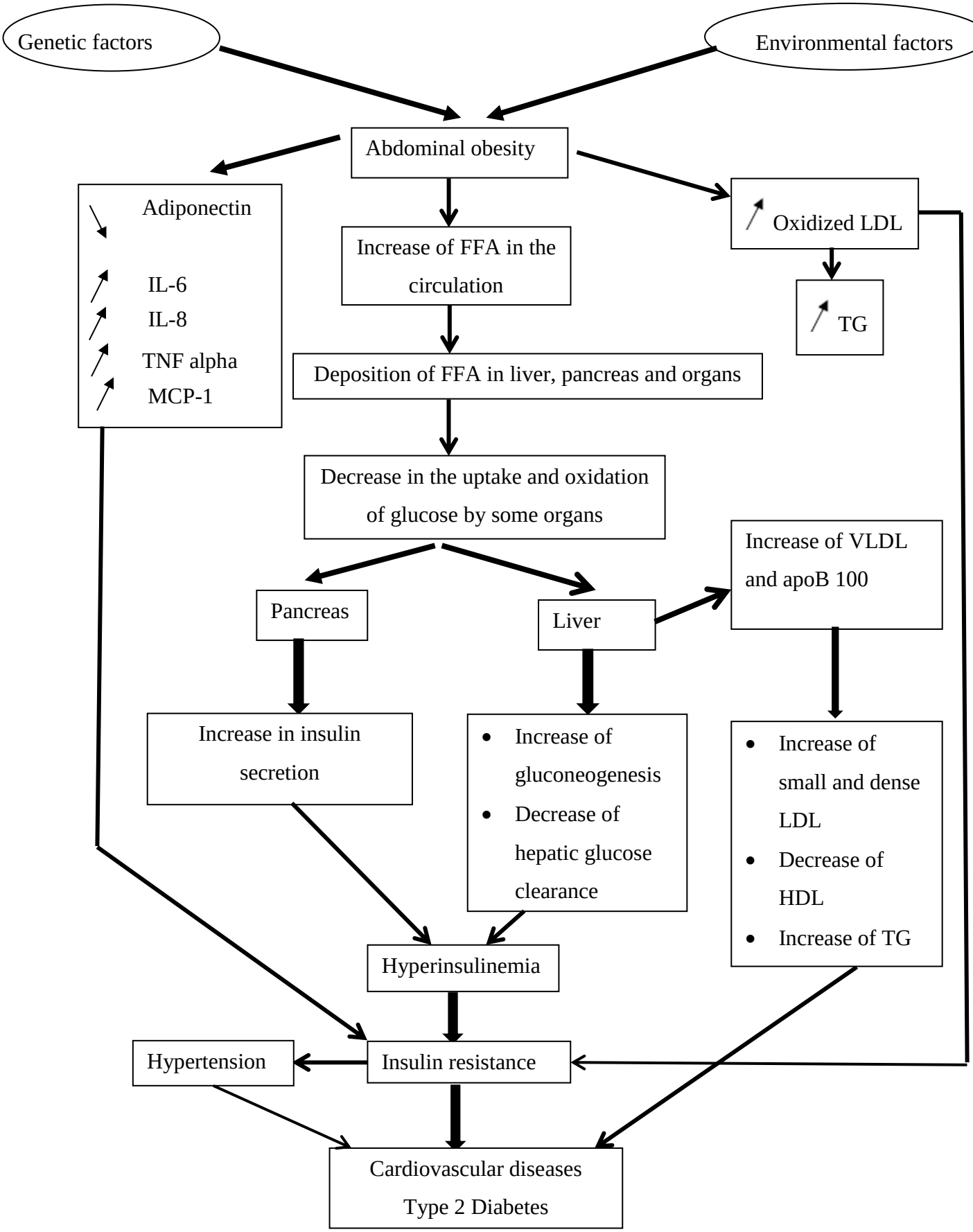



Figure 1.8: Pathophysiology and consequences of the metabolic syndrome

*IL: interleukin, TNF: Tumor necrosis factor, MCP-1:* monocyte chemoattractant protein-1, FFA: Free fatty acids

*d. Insulin signalling downstream pathways in obesity and insulin resistance*

Insulin is the central regulator of the metabolism of glucose and fat in the body. In addition to its role in inhibiting gluconeogenesis, lipolysis, and glycogenolysis, insulin induces proteogenesis, lipogenesis and glycogenesis (Figure 1.9). Furthermore, insulin increases the uptake of glucose by skeletal muscle through the increase in the translocation of the glucose transporter (GLUT4). Insulin also plays a significant role in cell growth and proliferation (Saltiel and Khan 2001).

Insulin receptor is a member of the family of tyrosine kinase receptors, and has numerous properties including secretion of insulin growth factor-1 (IGF-1), which is activated by insulin, and plays a role in regulating glucose homeostasis and insulin sensitivity (Clemmons 2004). The binding of insulin to insulin receptor leads to tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins (comprises six types of IRS labelled from IRS-1 to IRS-6), which stimulate the activation of insulin downstream signalling pathways like Ras-mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)–AKT/protein kinase B (PKB). These pathway cascades are responsible for most of insulin actions (Saltiel and Khan 2001), such as the increase in the translocation of GLUT4 (Wang et al. 1999). Obesity and IR have been reported to cause an alteration in the insulin signalling pathways. This has explained one of the mechanisms by which glucose metabolism is altered in obese individuals. A significant reduction in insulin-stimulated PI3K pathway has been noted in the muscle tissues of obese humans, while MAPK pathways have been shown to be normal (Cusi et al. 2000). It has been suggested that FFA lead to a reduction in PI3K activity (Saltiel and Khan 2001). In addition, in insulin resistant humans, it has been shown an impairment of AKT phosphorylation, suggesting a correlation between AKT and insulin sensitivity (Tonks et al. 2013).

Because of the major role of insulin in brain function, disruption of insulin-signalling pathways has been proposed to contribute to brain dysfunction. This may be due to a decrease in insulin uptake, leading to vascular damage caused by oxidative stress (Desideri et al. 2012).

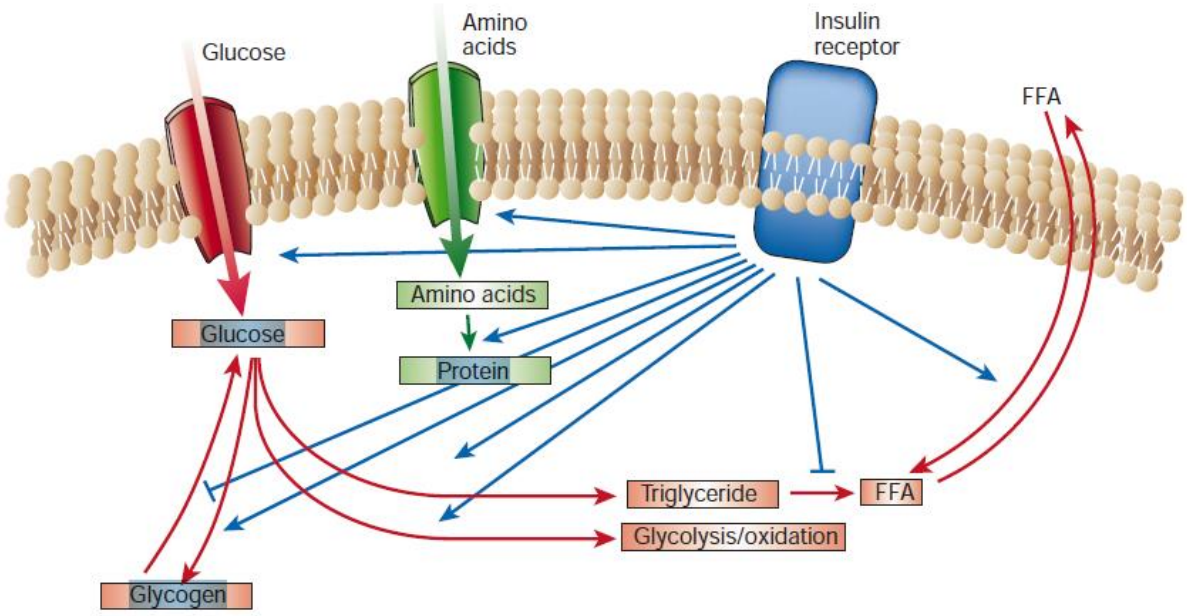



**Figure 1.9: Role of insulin in metabolic regulation**

(Saltiel and Khan 2001)

*FFA: Free fatty acids*
*Insulin is involved in the synthesis of carbohydrates, lipids and proteins and their storage. This hormone leads to an increase in the uptake of glucose, amino acids and fatty acids while inhibiting enzymes involved in the degradation of glycogen, protein and lipids. Insulin receptor is a mediator of these reactions (Saltiel and Khan 2001).*

### 1.1.5 Role of cortisol in obesity-related complications

Cortisol is a glucocorticoid counter-regulatory hormone secreted by the adrenal cortex in response to several stimuli such as stress and inflammation (Newton 2000). The role of this hormone involves the reduction of the insulin-dependent uptake of glucose, the decrease in insulin secretion by β-cells, as well as the increase in lipolysis and gluconeogenesis. Thus, cortisol acts as an antagonist for insulin (Rizza et al. 1982; Stulnig and Waldhausl 2004).

Stress caused by factors like smoking and alcohol may result in an increase in HPA (hypothalamic-pituitary-adrenal) axis activity, and a subsequent increase in cortisol levels (Andrews et al. 1999). However, stress can be induced by visceral obesity through the secretion of cytokines proportionally to abdominal fat. This results in the stimulation of HPA axis activity (Drapeau et al. 2003).

Cortisol is implicated in the pathology of insulin resistance through several mechanisms: Firstly, it increases hepatic glucose production and induces gluconeogenesis (Stulnig and Waldhausl 2004). In fact, glucocorticoids increase the hepatic gene expression of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme that catalyses the rate-limiting step in gluconeogenesis (Stulnig and Waldhausl 2004). Also, glucocorticoids inhibit GLUT4, resulting in the inhibition of cellular glucose uptake (Dimitriadis et al. 1997). Secondly, cortisol stimulates the secretion of the protein "resistin" by the adipose tissue (Qi and Rodrigues 2007), which may exacerbate IR (Pi-Sunyer 2007). Moreover, the elevated cortisol and HPA axis activity in abdominal obesity increase the mobilization of FFA, and lead to or exacerbate insulin resistance (Qi and Rodrigues 2007).

Glucocorticoid metabolism is mainly regulated by the enzyme 11β-HSD (11 beta hydroxysteroid dehydrogenase) which has two isoforms: 11β-HSD type 1 (11β-HSD 1), an enzyme that converts cortisone to cortisol using NADPH, and is highly expressed in the liver, central nervous system and adipose tissue; 11β-HSD type 2 (11β-HSD 2) an enzyme that oxidises cortisol to cortisone, and is essential to avoid the mineralocorticoid activity of cortisol. The enzyme 11 B-HSD2 is present in aldosterone-selective tissues such as kidneys (Van Uum et al. 1998; Agarwal et al. 1999).

As adipose tissue is the site of action of 11β-HSD1, the expansion of abdominal fat mass increases the enzyme activity, leading to an elevation in cortisol production (Stulnig and Waldhausl, 2004). The activity of this enzyme is particularly important in obesity, as it can be overexpressed in adipose tissue along with normal plasma cortisol levels (Engeli et al. 2004). Finally, the increased activity of 11β-HSD1 may inhibit the secretion of insulin from β cells, and cause a defect in the insulin-dependent uptake of glucose (Stulnig and Waldhausl 2004). Therefore, 11β-HSD1 plays a significant role in insulin resistance and glucose intolerance (Masuzaki et al. 2001), and may have a potential implication in the prevention/treatment of the metabolic syndrome.

### 1.1.6 Importance of prevention

There has recently been a large emphasis on the importance of prevention of chronic diseases before they occur, and its impact on reducing the burden on the health care system, and improving the nation's health and productivity. In fact, out of every two adults in the US, one person has a chronic disease, which is mostly preventable (CDC 2011a). Studies have shown that even a modest reduction in risk factors for chronic diseases may have a significant impact on decreasing their occurrence and/or their complications. Regarding obesity, a modest loss in body weight (5-10%) is associated with a significant decrease in risk factors for type 2 diabetes and CVD (Resnick et al. 2000; Wing et al. 2011). In addition, reducing IR preserves β-cell function, and prevents or delays the occurrence of type 2 diabetes (Chiasson and Rabasa-Lhoret 2004). As for blood pressure, studies have demonstrated that a slight decrease (5 mm Hg) in systolic and/or diastolic blood pressure is effective in decreasing cardiovascular risk by 20% (Glynn et al. 2002), the risk of stroke by 20% (Mclnnes et al. 2005), and mortality risk by 7% (Whelton et al. 2002). Finally, elevated lipid levels or dyslipidemia have been shown to be strong contributors to cardiovascular risk, and a small reduction of these levels may reduce the risk: A reduction of 1% in LDL cholesterol can decrease the risk of cardiovascular diseases by 2 % (Jia et al. 2010). Furthermore, according to the Framingham study, an increase in HDL levels by only 10 mg/dl leads to a reduction in cardiovascular risk by 2-3% (Ali et al. 2012). Therefore, any intervention that might have a slight significant impact on the above mentioned parameters can possibly improve the general health, and help in decreasing the occurrence of chronic diseases.

## 1.2 Polyphenols: Overview

### 1.2.1 Definition and properties

Polyphenols (PPs) constitute a class of phytochemicals widely available in plants (Stevenson and Hurst 2007), in which they are known to exhibit numerous functions including protection from solar UV radiation and free radicals generated during photosynthesis, prevention of insect feeding, as well as anti-microbial and anti-

fungal functions. PPs also play a role in the determination of the colour of the leaves, fruits and flowers (Gould and Lister 2006). PPs are not essential nutrients, therefore are not required for growth and reproduction. PPs have been frequently described as non nutrients (Martin and Appel 2010).

Polyphenols are largely abundant in the human diet, and their average daily intake is estimated to be around 1 g/day (Scalbert and Williamson 2000). The studies of diet diaries in the UK showed that the mean intake of total polyphenols ranges between 780 mg/day for females and 1058 mg/day for males, with flavonoids consisting of 20-25% of the polyphenol intake (Stevenson and Hurst 2007). However, individual preferences might influence this intake (Manach et al. 2004). PPs are extensive in chocolate, tea, coffee, wine, olive, dry legumes, and some vegetables (like lettuce and cabbage) and fruits (like apples and berries) (Manach et al. 2004; D'archivio et al. 2007).

Polyphenols have gained a particular interest over the past two decades as positive contributors to human health, mainly because of their antioxidant properties and their possible implication in the prevention of several diseases such as CVD, cancer, and neurodegenerative diseases, all known to be related to oxidative stress (Manach et al. 2004; D'archivio et al. 2007). The antioxidant properties of polyphenols rely in their potential reducing capacity, and their ability to bind to free radicals and neutralize them (Scalbert and Williamson 2000), resulting in the reduction in the development of atheromatous lesions (D'archivio et al. 2007). Polyphenols have been suggested to improve insulin sensitivity (De Bock et al. 2013), to function as vasodilators (Kim 2007) and to exert positive effects on the metabolism of lipids (by increasing fat oxidation) (Dulloo et al. 1999) and carbohydrates (by inhibiting digestive enzymes (Ishikawa et al. 2007), and decreasing glucose absorption (Johnston et al. 2005)). PPs have been also implicated in several biological functions such as protecting the skin (Nichols and Katiyar 2010), improving the immune system (Safonova et al. 2001; Baeza et al. 2010), decreasing platelet aggregation (Murphy et al. 2003) and reducing inflammation by inhibiting cyclooxygenase 2 (COX-2) (O'Leary et al. 2004), and lipoxygenase (Sadik et al. 2003) enzymes. The latter enzymes are implicated in inflammation through the secretion of prostanglandins.

Despite their high intake in the diet, the maximum plasma concentration of polyphenols is usually in the micromolar and nanomolar ranges. This is due to the low bioavailability of PPs, which is dependent on their chemical structure. In fact, the structure is a better determinant of the bioavailability of polyphenols than their concentration (D'archivio et al. 2007). Consequently, not all pps can be attributed beneficial properties (Scalbert and Williamson 2000). Recognizing the different types and structures of pps and their bioavailability will help to determine the PPs that are more likely to exert a favourable effect on human health. This will provide a background for understanding the mechanism of action of cocoa polyphenols, which are the ones of interest in this thesis.

### 1.2.2 Types of polyphenols, chemical structure and food sources

PPs are vastly diverse with a complex chemical structure (D'archivio et al. 2007). Around 8000 structures of PPs have been already identified (Martin and Appel 2010). PPs are characterized by having at least one six-carbon aromatic ring and not less than two phenolic hydroxyl groups (Stevenson and Hurst 2007), and are classified according to their chemical structure (such as the number of phenol rings, the ways of binding between aromatic rings) into four groups: Flavonoids, Lignans, Stilbenes and Phenolic acids (Manach et al. 2004) (Figure 1.10)

#### 1.2.2.1 Flavonoids

Flavonoids are the most abundant type of PPs in foods (Salbert and Williamson 2000), and more than 4000 flavonoids have been recognized in plants (D'archivio et al. 2007). Flavonoids consist of 2 aromatic rings bound by a three carbon chain which form an oxygenated heterocycle, and are subdivided into 6 classes according to the degree of oxidation of the oxygen heterocycle into flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Manach et al. 2004) (Figure 1.11).

*a. Flavonols*

Flavonols consist of a double bond between Carbon 2 and Carbon 3, and a hydroxyl group in the Carbon 3 position (D'archivio et al. 2007). Flavonoids are stimulated by light, hence the flavonol content of plants depends on sunlight exposure (Manach et al. 2004), which results in differences in the concentration of fruits on the same tree and even between different parts of the same fruit (D'archivio et al. 2007). Flavonols consist of quercetin, kaempferol, myricetin and isorhamnetin, and are widely present in onions, kale, red wine, teas, and blueberries (Manach el al. 2004; D'archivio et al. 2007).

*b. Flavones*

Flavones are less common than other flavonoids. They have a double bond between Carbon 2 and Carbon 3 (D'archivio at al. 2007), and consist of apigenin and luteolin. Flavones are available in parsley, thyme, celery, hot peppers, cereals (Manach el al. 2004), and the skin of citrus fruits (Shahidi and Naczk 1995).

*c. Isoflavones*

Isoflavones are flavonoids that have a structural similarity with estrogen. They are classified as non steroids, and are able to bind to estrogen receptors. They consist of daidzen, genistein and glycitein, which are mainly present in soya and its derived products. Due to their estrogenic properties, isoflavones have been implicated in the prevention of breast cancer and osteoporosis (Adlercreutz and Mazur 1997). These polyphenols are sensitive to heat and are often hydrolysed to glycosides during industrial processing and storage, such as during the production of soy milk (D'archivio at al. 2007).

*d. Flavanones*

Flavanones are characterized by a saturated three-carbon chain and an oxygen atom in the Carbon 4 (D'archivio at al. 2007). They are usually glycosylated by a disaccharide, and are widely available in citrus fruits like oranges, lemons and

grapefruit (Manach et al. 2004). The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons. The flavonone content is particularly high in the solid part of citrus fruits, especially in the white spongy portion and the membranes that separate the segment (D'archivio at al. 2007).

*e. Anthocyanidins*

Anthocyanins are pigments mostly abundant in fruits, and are responsible for the pink, red, blue, and purple colour of berries (Clifford 2000). They are characterized by a positive charge on the heterocyclic ring (Alkema and Spencer 1982).
Also called anthocyanidins, anthocyanins are mostly available in berries, cherries, blackcurrant and grapes, but also in vegetables such as cabbage, onions and aubergines. Anthocyanins are mainly found in the skin of fruits, except for some red fruits (cherries and strawberries), in which they are found in the flesh. Red wine is also an important source of anthocyanins. Food content of anthocyanidins is correlated with the colour intensity and the degree of ripeness of fruits. (D'archivio et al. 2007). Anthocyanidins consist of cyanidin (the most common food source of anthocyanidins), delphindin, malvidin, pelargonidin, peomidin and petunidin (Manach et al. 2004; Jakobek et al. 2007).

*f. Flavanols*

Flavanols are one of the most abundant types of flavonoids in the diet (Scalbert and Williamson 2000). They contain a saturated three-carbon chain with a hydroxyl group in the Carbon 3 (hence the nomination flavan-3-ols). They are available as monomers (catechins), dimers (theaflavins) and polymers (thearubigins and proanthocyanidins). The dimers and polypmers are building blocks of monomers (Cooper et al. 2008). Catechins consist of catechin, epicatechin, epicatechin gallate and epigallocatechin gallate, which are widely available in teas, especially green and white tea (Scalbert and Williamson 2000; Higdon 2005). Epicatechin is abundant in cocoa and chocolate, as well as in berries, grapes and apples, but to a lesser extent (Manach et al. 2004). Theaflavins and thearubigins are particularly present in black and oolong teas. As for proanthocyanidins (also known as procyanidins or condensed tannins), they are abundant in chocolate, red wine, apples, berries and red grapes,

and give the astringency to fruits and beverages, and the bitterness to chocolate. Proanthocyanidins usually occur along with catechins in foods (Santos-Buelga and Scalbert 2000). Unlike other types of flavonoids, flavanols are not glycosylated in foods (Zhu et al. 1997).

Catechin and epicatechin have two chiral centers each: (+)- catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin, respectively (Figure 1.12). The enantiomers (+) – catechin and (-)-epicatechin are predominant in foods, while the two other epimers rarely occur in nature (Kofink et al. 2007). Proanthocyanidins occur as oligomers (with degree of polymerization between 2-10) and polymers (degree of polymerization > 10) in plants (Glinski et al. 2012) (View Figure 1.13 on the chemical structure of the most common procyanidins).

#### 1.2.2.2 Lignans

Are only found in significant amounts in flaxseed and flaxseed oil, and are mostly present in nature in their free form. Linseed is the most abundant dietary source of lignans (D'archivio et al. 2007). Lignans are also present in oleaginous seeds, and some vegetables (like garlic and carrots), and fruits (like pears and prunes) (Thompson et al. 1991). Lignans are mainly known for their estrogenic properties (Scalbert and Williamson 2000), and their use in cancer chemotherapy and some medications (Saleem et al. 2005).

#### 1.2.2.3 Stilbenes

Human diets provide low quantities of stilbenes. The most common type is resveratrol which has been reported to have anticarcinogenic properties. Resveratrol is found particularly in the skin of red grapes, and consequently in red wine and grape juice (Manach et al. 2004; D'archivio et al. 2007).

#### 1.2.2.4 Phenolic acids

Are divided into two classes: hydroxybenzoic acids and hydroxycinammic acids (D'archivio et al. 2007)

*a. Hydroxybenzoic acids*

Are divided into gallic acid and components of tannins (gallotannins and ellagitannins), which are rare in the edible parts of plants, except for some red fruits, onions and black raddish (Shahidi and Naczk 2005). Tea contains a considerable amount of gallic acid (Tomás-Barberán and Clifford 2000). Due to their limited occurrence in plants, limited research has been carried out on these acids.

*b. Hydroxycinnamic acids*

Are more common than hydroxybenzoic acids, and have 4 subclasses: p-coumaric, caffeic, (the most abundant phenolic acid), ferulic, and sinapic acids. Caffeic acid is usually combined to quinic acid to form chlorogenic acid, which is abundant in coffee (Clifford 2000). These phenolic acids are also present in fruits such as blueberries, apples and cherries (Macheix et al. 1990).

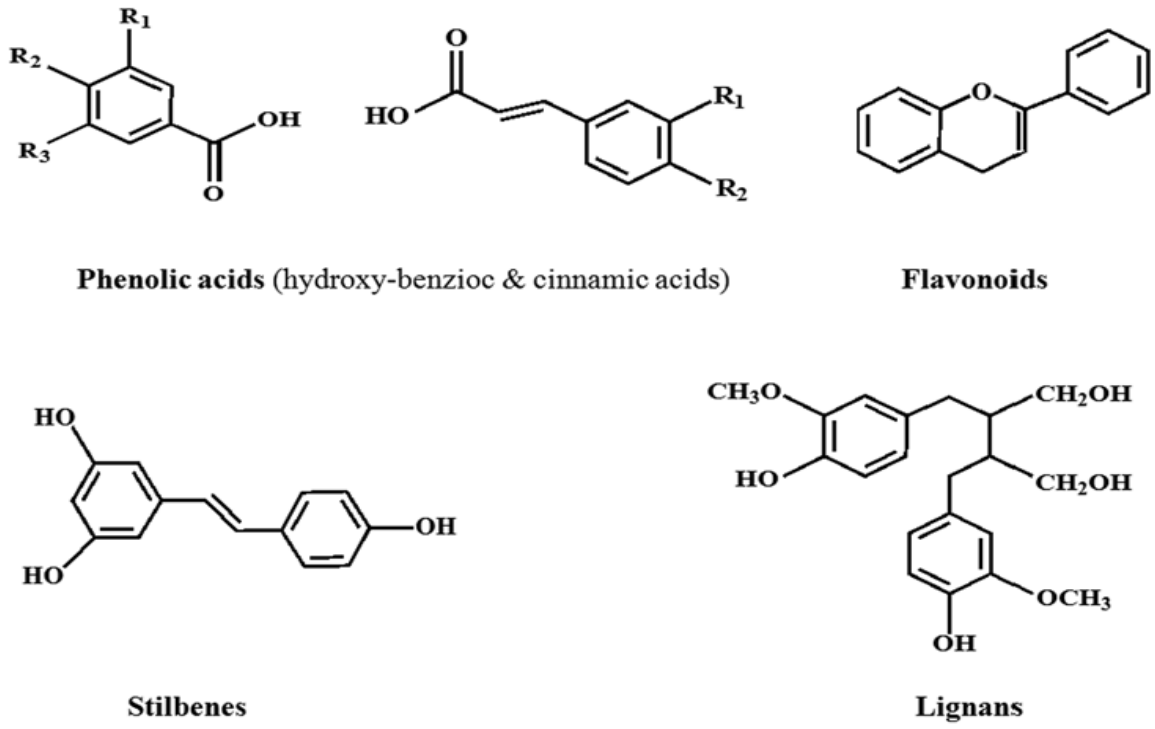



**Figure 1.10: Chemical structure of polyphenols**
(Pandey and Rizvi 2009)

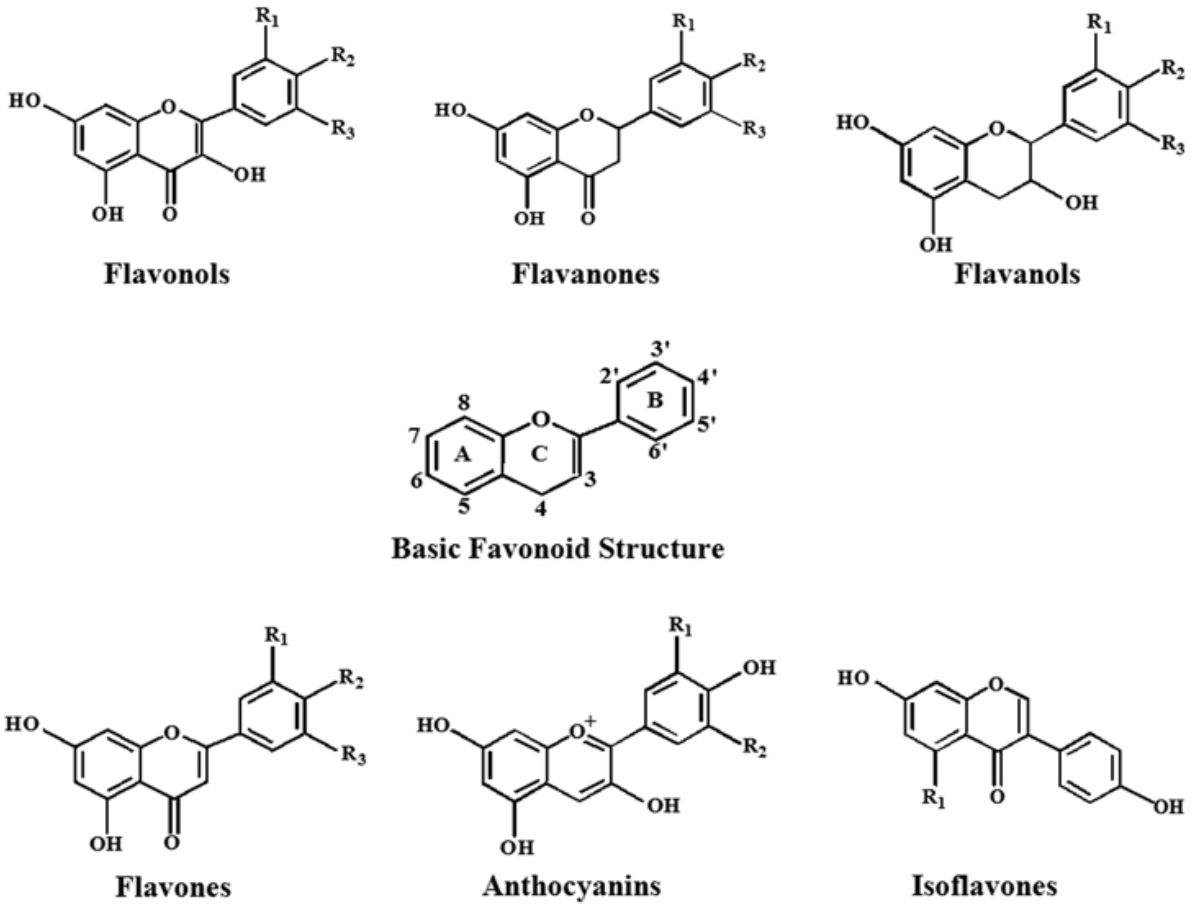



**Figure 1.11: Chemical structure of flavonoids**

(Pandey and Rizvi 2009)

### 1.2.3 Factors affecting polyphenol content in foods

Several factors may affect the polyphenol content in foods. Different varieties of the same fruit contain various amounts of polyphenols (Manach et al. 2004). Hence, the amount or percentage of PPs in foods is usually estimated. In addition, environmental factors such as climate (like degree of sun exposure and rain), agronomy (like type of culture, yield of fruit per tree and degree of ripeness) and sunlight exposure exert an important influence on the polyphenol content of fruits and vegetables (Macheix et al. 1990; Van der Sluis 2001). For instance, the degree of

ripeness may decrease the proportion of some PPs like phenolic acids, but increase the concentration of anthocyanins (Shahidi et al. 1997). Storage at room temperature may also lower polyphenol content in foods due to the fact that PPs can be readily oxidised (Manach et al. 2004), whereas this is not the case during cold storage (Burda et al. 1990). Furthermore, the polyphenol content is influenced by the method of culinary preparation. Cooking and peeling of vegetables and fruits may cause a substantial loss of polyphenols in foods (Crozier et al. 1997; Manach et al. 2004). Lastly, industrial food processing may result in a decrease in polyphenol content as during the production of tomato products (Takeoka et al. 2001), or an increase in this content as during the production of beer (due to ethanol which increases the extraction of polyphenols) (Leitao et al. 2011).

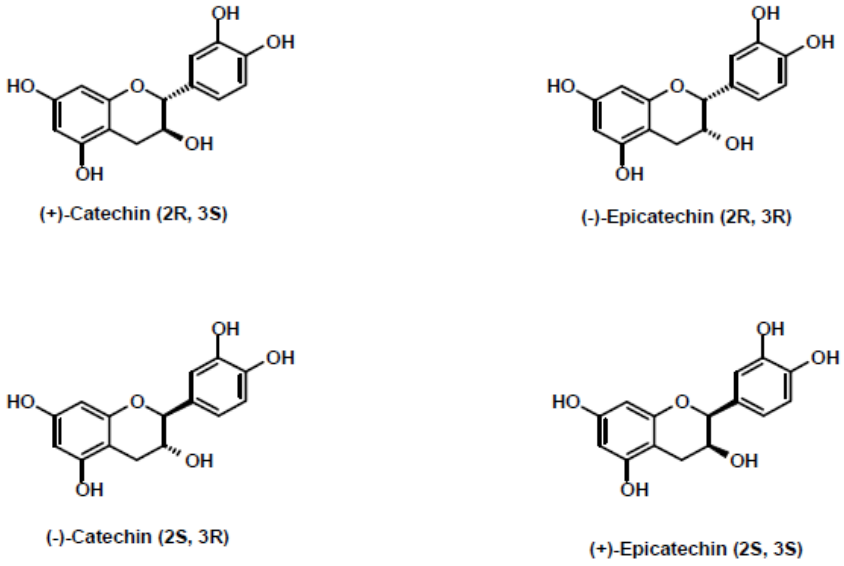



**Figure 1.12: Chemical structure of catechin and epicatechin enantiomers**
(Adapted from Kofink et al. 2007)
*S stands for left and R stands for right*

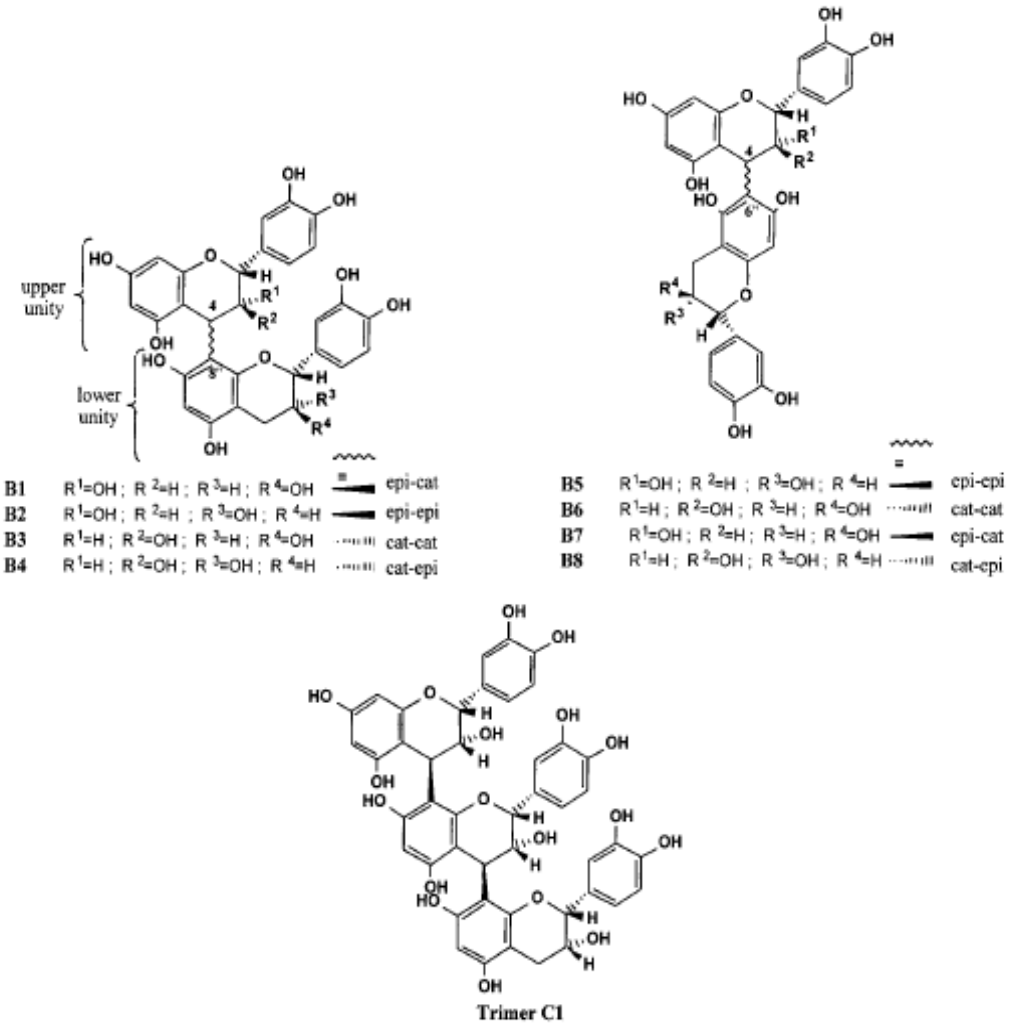



**Figure 1.13: Chemical structure of common procyanidins**

*(Adapted from De Freitas and Mateus 2001)*
*epi: epicatechin, cat: catechin. Figure represents procyanidins dimers (B1-B8) and trimer C1. The latter is an epicatechin trimer.*

### 1.2.4 Metabolism of polyphenols: from absorption to elimination

Because they are extremely diverse, the metabolism of PPs differs with types and within subtypes and is not yet fully elucidated in the human body. About 5-10% of polyphenols are absorbed in the small intestine (the aglycones), and the rest (occurring in the glycosylated form) escape the intestinal absorption and pass into the colon where they are broken down by the colonic micro-flora, and transformed into metabolites. A small fraction of these metabolites is then absorbed (Clifford 2005).

Most of flavonoids (with the exception of flavanols) occur in the glycosylated form (D'archivio et al. 2007), which pass through the intestine (Manach and Donovan 2005) and are then transported into the colon where they are hydrolysed by the colonic flora into derivatives of benzoic acids (D'archivio et al. 2007). The concentration of polyphenol metabolites can reach hundreds of micromoles/liter in the colon (Manach et al. 2004). It could be therefore suggested that many of the biological effects of polyphenols are due to their metabolites produced in the colon. These metabolites have been involved in the protection from oxidative stress (Manach et al. 2004), and in the inhibition of platelet aggregation (Rechner and Kroner 2005). Polyphenol metabolites in the colon have been also reported to increase the number of gut microbiota; thus they can exert a beneficial role in the gut (Parkar et al. 2008). For instance, administration of red wine polyphenols to rats increased the faecal bacterial composition of Lactobacillus and Bifidobacterium spp, which resulted in a decrease in inflammation and oxidative stress (Dolara et al. 2005). Also, in humans, the administration of cocoa (494 mg of flavanols) for 4 weeks increased bifidobacteria and lactobacilli counts in the fecal samples at the end of the intervention. Lactobacilli counts were inversely proportional to CRP levels in this study (Tzounis et al. 2011). Moreover, an increase in bifidobacteria was noted after blueberry drink supplementation daily for 2 weeks (Vendrame et al. 2011). These examples highlight the potential role of polyphenols in increasing specific bacteria counts in the colon, and might provide an explanation for their potential lowering effects on inflammation and oxidative stress, despite their low concentrations in the blood.

After absorption, PPs undergo series of conjugation reactions, including methylation glucuronidation and/or sulfation in the liver, resulting in metabolites that are different from the ones originally present in foods (D'archivio et al. 2007). Conjugation is mainly carried out as a detoxication process in order to facilitate the elimination of polyphenols and limit their toxic effects. This results in small concentrations of polyphenols reaching the plasma (Manach et al. 2004). Polyphenol metabolites circulate in the blood bind to proteins, mainly albumin. Hence, it is likely that the cellular uptake of metabolites depends on the amount of PPs bound to

albumin (D'archivio et al. 2007). PPs are then taken by tissues, and subsequently eliminated in addition to their metabolites in the urine and bile. This elimination is dependent on the level of conjugation, so that highly conjugated metabolites are eliminated in the bile (like genistein and epigallocatechin gallate), and less conjugated ones are eliminated in the urine (like flavanones and isoflavones) (Manach et al. 2004; D'archivio et al. 2007). A simplified explanation of the metabolism of polyphenols in the human body is presented in Figure 1.14.

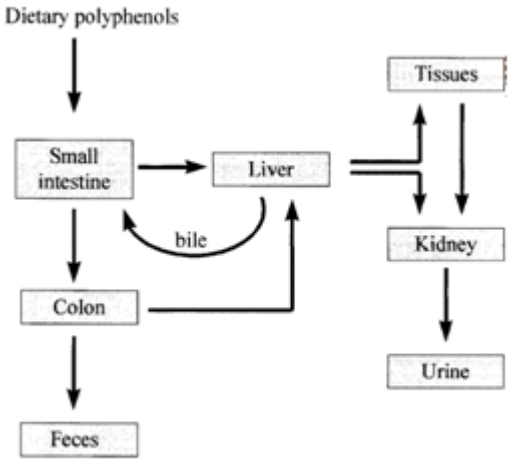



**Figure 1.14: Possible pathways of polyphenols in the human body**
(Scalbert and Williamson 2000)

### 1.2.5 Bioavailability of polyphenols in the human body

Recognizing the bioavailability of polyphenols is highly important, as some types of PPs are abundant in the diet, but are poorly bioavailable because of their reduced intestinal absorption or their rapid elimination which makes them less likely to accumulate in the plasma (Manach et al. 2005). Another issue in estimating the bioavailability of PPs remains in the fact that some polyphenol metabolites are still unknown, which renders it more difficult to evaluate the biological activity of PPs in the body (D'archivio et al. 2007). These metabolites might actually be the true active components rather than the native polyphenols (Kroon et al. 2004).

The bioavailability of polyphenols, apart from being dependent on the chemical structure, is also subject to intra-individual variety. This is probably due to the different composition of the colonic microflora, which may result in differences in the metabolism of PPs between individuals (D'archivio et al. 2007). Other factors, such as food matrix, efficiency of binding to albumin and extent of excretion in urine and bile may also affect bioavailability (Ali et al. 2014). It has been estimated that the bioavailability of PPs range between 2 - 20% (Hu 2007).

Data collected from 97 bioavailability studies provided valuable information regarding polyphenol bioavailability (Manach et al. 2005). Among flavonoids, anthocyanins have a poor bioavailability despite their high intake in certain diets, mainly because of their low absorption rate and their rapid elimination. In addition, the bioavailability of flavonols is usually limited in the diet, and its bioavailability is dependent on the food source (for instance, onions contain more bioavailable quercetin than apples). As for flavanols, catechins are highly abundant in the diet and are generally well absorbed (except for galloylated tea catechins). While proanthocyanidins are poorly absorbed, data on flavanone bioavailability is limited. Finally, isoflavones have been shown to be the best bioavailable among polyphenols, yet their intake in the human diet is limited to citrus fruits and soja (Manach et al. 2005). An example of the bioavailability of various types of flavonoids or foods containing flavonoids based on human studies is presented in Table 1.3.

**Table 1.3: Bioavailability of different types of flavonoids**

| Polyphenols | Source | Quantity of polyphenols (mg) | Maximum concentration (µM) |
|---|---|---|---|
| **Anthocyanins** | | | |
| Cyanidine 3-glucoside | Orange juice (1 L) | 71 | 0.002 |
| Malvidin 3-glucoside | Red wine (500 ml) | 68 | 0.001 |
| **Flavanols** | | | |
| Epigallocatechin gallate | Green tea infusion(5g) | 105 | 0.13 - 0.31 |
| Catechin | Red wine (120 ml) | 34 | 0.072 |
| Epicatechin | Chocolate (80g) | 137 | 0.26 |
| Catechin | Pure compound | 0.36 mg/kg/bw | 0.14 - 0.29 |
| Epigallocatechin gallate | Pure compound | 50-1600 | 0.28 - 7.4 |
| Epigallocatechin gallate | Green tea extract | 110-328 | 0.26 – 0.7 |
| Epicatechins | Black tea | 140 | 0.34 |
| Procyanidin B1 | Grapeseed extract | 18 | 0.011 |
| **Flavanones** | | | |
| Hesperidin | Orange juice | 61 | 0.48 |
| Hesperidin | Pure compound | 135 | 2.7 |
| Naringenin | Grapefruit juice | 199 | 5.99 |
| **Flavonols** | | | |
| Quercetin | Apples | 107 | 0.3 |
| Quercetin | Onions | 100 | 7.6 |
| **Isoflavones** | | | |
| Genistein | Soy milk | 108 | 0.47 |
| Genistein | Pure compound | 50 | 0.76 |
| Daidzen | Pure compound | 50 | 1.26 |
| Daidzen | Soy nuts | 9.8 – 39.2 | 0.59 – 2.21 |

(Adapted from D'archivio et al. 2007)
*Bw: body weight. Data based on human studies.*

### 1.2.6 Toxicity of polyphenols

Despite their beneficial role in human health, high intakes of polyphenols have been suspected to have pro-oxidant effects. Because of their low bioavailability which plays a regulatory role, PPs are unlikely to cause side effects when consumed in foods, and are expected to exhibit antioxidant properties. It is mainly the pharmacological doses that may cause adverse effects. Also, supplementing foods with polyphenols can be problematic (Martin and Appel 2007). Dietary supplements of polyphenols usually contain high levels ranging from 1 g of flavonoids, 20 mg resveratrol and 300 mg of quercetin per capsule, and the recommendations commonly range from 1- 6 capsules per day. This leads to intakes that are 100 times higher than the usual western polyphenol intake (Mennen at al. 2005).

Animal studies have related high doses of green tea polyphenols to several side effects like severe kidney and liver damage, haemorrhagic lesions in stomach and intestine, and multi-organ toxicity (Bonkovsky 2006). In addition, some human case studies showed hepatotoxicity related to tea dietary supplements enriched in polyphenols, and an increase in liver enzymes and in serum bilirubin levels which went back to normal after discontinuation of the supplement (Martin and Appel 2010). Furthermore, isoflavones such as genistein and daidzen, have been potentially involved in the occurrence of breast cancer due to their estrogenic like effects and their role in tumour growth (Messina and Loprinzi 2001).
High doses of flavonoids have been reported to cause clastogenicity and chromosome translocation in human cell line studies, due to the fact that flavonoids act as inhibitors of topoisomerase II (an enzyme that plays an essential role in cellular DNA transcription and replication) (Constantinou et al. 1995). Polyphenols may also affect nutritional status; the most eminent example is the inhibitory effect of tea polyphenols on heme iron absorption (Nelson and Poutler 2004).

Data are still lacking regarding the doses of polyphenols supposed to cause toxicity effects. Hence, further research particularly in animal toxicology testing, is needed. In view of these studies, more attention should be given to the dose of PPs used in supplements or in polyphenol-enriched foods.

## 1.3 Cocoa / Dark chocolate

Chocolate, a product of cocoa, is a largely consumed food in the western society. It is estimated that the consumption of chocolate is around 10 Kg per person per year in the UK (CAOBISCO 2013). Chocolate constitutes the snack of choice for 40% of women in the US (Rozin et al. 1991). Chocolate is also a highly craved food: It has been reported that it constitutes 59 % and 91 % of the food cravings in American university males and females, respectively (Osman et al. 2006). Yet, chocolate does not qualify for addiction, due to the lack of evidence of physical dependency towards any of the chocolate components (Rogers and Smit 1999). The popularity of chocolate and its frequent consumption made it the target of many research studies, due its favourable effects, and to the significant role it may exert on improving the population's health. Therefore, studies that elucidate these effects are/were needed to guide the consumers' choice.

### 1.3.1 History

Cocoa and chocolate are derived form cacao beans, the fruit seeds of "Theobroma cacao" tree, which stands for "the food of the Gods" (Lima et al. 2011; Lima et al. 2012). The tree is originally a tropical tree from the rain forest of Amazon, but was brought by the human activity to the rest of the world (Kris-Etherton and Keen 2002). The term "cacao" originated from the Olmec peoples, and was named "Cacahuatl" by the Mayans who considered it to be a divine food (Dillinger et al. 2000; Lippi et al. 2009).

The cultivation of cocoa and the tradition of drinking chocolate might have first originated in the Maya Lowlands in Mesoamerica as early as 600 years BC. The Mayans and the Aztecs used to froth liquid chocolate to produce foam, which was the most appealing part of the drink (Hurst et al. 2002; Lippi et al. 2009). Another research suggested that solid and liquid chocolate foods were consumed by the inhabitants of the North of Honduras by 1200-1100 BC. However, more recent data indicate that chocolate was consumed by the pre-Olmec and the Mokaya peoples as early as 1750 BC and 1900 BC, pushing back the use and cultivation of cacao by around 700 years (Powis et al. 2007).

Cocoa was claimed to be aphrodisiac. It was rumoured that Montezuma, the Aztec emperor, used to have a cocoa drink before visiting his wives (Dillinger et al. 2000). Cocoa was also claimed to make people happy and strong (Steinberg et al. 2003), and was restricted to high social classes (Lippi et al. 2009).

The Spanish brought cocoa to Europe after the conquest of South and North America during the 16$^{th}$ century (Bearden et al. 2000). The conquistador Hernando Cortes wrote a letter to the Spanish crown describing cocoa as having miraculous properties, claiming that one cup allows a soldier to have enough strength to march for a whole day (Lippi et al. 2009). Cocoa was traditionally consumed as a cold drink, without any sugar added, and often mixed with starch and spices. Sugar was subsequently added to it (Lippi et al. 2009).

Europeans tended to consider chocolate sinful, the same way was every black, spicy and hot food. Due to its euphoric effects, the church considered drinking chocolate as a breaking of the fast, and its use was only restricted to medical reasons (Lippi et al. 2009).

The production of cocoa powder only started in the 18$^{th}$ century, followed by the production of solid chocolate then milk chocolate. These products were then consumed by all society classes (Borchers et al. 2000). From the sixteenth till the early twentieth century, more than 100 medicinal uses were attributed to cocoa and chocolate, such as treating anemia, fever, tuberculosis, gout, kidney stones, and curing some tumours. Furthermore, chocolate was used in the conditions of haemorrhoids, hypochondria, poor breast milk production and fatigue (Dillinger et al. 2000; Lippi et al. 2009). It was believed that cocoa relieves heart pain, strengthens the heart, comforts the liver, helps in digestion and induces sleep (Steinberg et al. 2003; Lippi et al. 2009). Chocolate was also used to help wasted patients to gain weight (Dillinger et al. 2000).

Chocolate is considered more than a snack or beverage: it is a connection from 3000 years ago to the present, and is an important part of the history. The current society has a high demand for cocoa products, and sees chocolate as a luxury (McShea et al. 2008), energizing, healthy but sometimes fattening and unhealthy food (Dillinger et al. 2000).

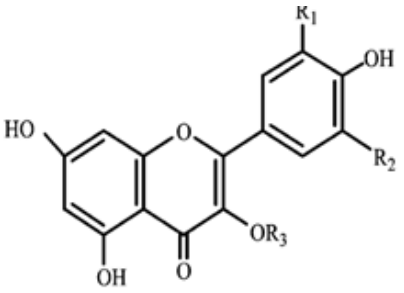
### 1.3.2 Cocoa and dark chocolate composition

Dark chocolate (DC) contains high amounts of cocoa which usually range between 50-85%, while white chocolate contains 20-30% of cocoa (Ried et al. 2012). DC contains over 500 different components (Powis et al. 2007), including theobromine (significant concentrations), caffeine (small amounts), amino acids, sour tasting compounds (like acetic acid), sweet tasting compounds (like sucrose which is naturally occurring but also added to increase palatability) and polyphenols. Cocoa is also a source of fibre (mainly insoluble), which remains from the processed bran (contains initially a high amount of fibre) (Cooper et al. 2008). DC consists of cocoa liquor (not less than 35% by weight (FDA 2013)) and cocoa butter (makes about 50 – 57 % of the dry weight of cacao beans) (Hannum and Erdman 2000). The cocoa liquor consists of the non fat cocoa solids, and comprises polyphenols (as well as vitamins, minerals and fibre), whereas cocoa butter contains no polyphenols (Cooper et al. 2008). Cocoa butter contains 33% of stearic acid, 33% of oleic acid and 25% of palmitic acid (USDA 2011a). Although a saturated fatty acid, stearic acid is considered neutral on cholesterol and has been therefore considered nonatherogenic (Bracco 1994).

As for polyphenols, cocoa beans contain 6-8% of polyphenols by dry weight (Bravo et al. 1998). Cocoa products are considered, along with tea, the major contributors to polyphenol intake in the western diet (Taubert et al. 2007). Cocoa polyphenols mainly consist of flavanols (including epicatechin, catechin and procyanidins) and quercetin (flavonol). Other polyphenols such as anthocyanins, phenolic acids and stilbenes have also been reported to be present in small amounts (Hurst et al. 2008). Flavanols are responsible in addition to theobromine and caffeine of the astringent taste of chocolate (Stark et al. 2006; McShea et al. 2008). Flavanols are highly available in DC (Chaitman et al. 2004). It is estimated that commercial DC contains 5 times more flavanols that milk chocolate (Miller et al. 2008). The amount of epicatechin and catechin in DC is approximately 20 times higher than that of tea, and four times higher than milk chocolate (Chaitman et al. 2004). Flavanols monomers consist of (+)catechin and –(-)epicatechin (Porter et al. 1991). The isomer –(-) epicatechin is the main flavanol, and constitutes up to 35% of the polyphenol content

of cocoa (Cooper et al. 2008). Procyanidins are usually present as dimers (B1, B2, B3, B4, B5), trimers (C1) and tetramers (Cinnamtannin A2) in chocolate, and are linked by 4→6 or 4→8 bounds (Porter et al. 1991; Natsume et al. 2000). Procyanidins dimers type B2 are the most available (Galleano et al. 2009), and it has been reported that other procyanidins polymers exist in unfermented cocoa beans (Kelm et al. 2006). As for quercetin, limited studies have quantified its content in cocoa/chocolate, due to its low concentration compared to flavanols. Several forms of quercetin have been identified in cocoa, such as quercetin aglycone (quercetin) and the glycosides quercetin-3-O-glucoside (isoquercitrin), quercetin-3-*O*-glucuronide and quercetin-3-O-arabinoside. The latter two forms have been shown to be the most prevalent in cocoa (Andres-Lacueva et al. 2008) (Figure 1.15).
This high amount of PPs provides cocoa/DC a high antioxidant activity, which together with the high concentration of lipids, extends the shelf-life of these products (McShea et al. 2008). Interestingly, PPs and flavanols can be stable for over a year in commercial cocoa products, and it has been shown that pure cocoa powder retains a high level of antioxidant capacity for almost 80 years (Hurst et al. 2009).

Nonetheless, the amount of polyphenols in cocoa/DC, and consequently the antioxidant activity, depends on the variety and ripeness of cacao beans (Ried et al. 2012), as well as the origin, agricultural practices, and handling following the harvesting of cacao beans (Chaitman et al. 2004). However, the method of processing is the major determinant of the final polyphenol content. Hence, it is very likely that the amount of flavanols in two different types of chocolate with equal cocoa concentrations markedly differ from one chocolate to another in the market depending on the level of processing (McShea et al. 2008; Ried et al. 2012).




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Quercetin | OH | H | H |
| Quercetin-3-glucoside (isoquercitrin) | OH | H | glc |
| Quercetin-3-arabinoside | OH | H | arb |
| Quercetin-3-glucuronide | OH | H | gluc |

**Figure 1.15: Chemical structure of the major forms of quercetin in cocoa/chocolate**

(Adapted from Andres-Lacueva et al. 2008)
*Glc: glucoside, arb: arabinoside, gluc: glucuronic acid*
*R1, R2 and R3 represent the monovalent groups*

### 1.3.3 Cocoa and chocolate production

The three main varieties of theobroma cacao are: Criollo, Forastero, and Trinitario (the most common). Cocoa has a significant genetic diversity, and more than 14000 varieties of cocoa have been suggested. However, the distinction between one type to another is minimal, as cocoa products contain a mixture of these varieties (Turnbull et al. 2004).

Chocolate production has always remained undisclosed, and little has been said about the details of its manufacturing. The process results in rendering the cacao bean more palatable, and in producing different flavours by altering the physical and chemical properties of cacao beans (McShea et al. 2008). The processing of chocolate is also related to national preferences (Egan et al. 2010).

Since processing is a crucial stage in the determination of the final polyphenol content in cocoa/chocolate, it is therefore important to understand the different steps involved in the production of these cocoa products, and to identify the phases that mostly affect the polyphenol content of the end product. The processing of cocoa and dark chocolate is presented in Figure 1.16.

The process starts by fermentation of the dried beans (for up to 7 days), resulting in the breakdown of the seed. This step is followed by drying, cleaning and subsequently roasting; the latter process permits the loss of the shell, and the development of different flavours through several reactions like Maillard and

caramelisation of sugars. The roasted beans next undergo winnowing; a subsequent step that removes the husk of the cacao bean, and breaks the latter into small particles called cocoa nibs, which constitute the first palatable produce of chocolate processing. Cocoa nibs then undergo milling in order to develop more flavours and to yield the chocolate liquor (McShea et al. 2008).

Depending on the end product, several ingredients (sugar, milk powder and some additives) are then added to the liquor, and the quantities vary considerably. For instance, in milk chocolate, there is an addition of a significant amount of milk powder and sugar, whereas in the manufacture of dark chocolate, there is only addition of sugar and cocoa butter and additives at this stage (Minifie and Chem 1989). This step is followed by further refining and mixing to ensure the particle sizes are homogenously distributed inside the product. Subsequently, a heating step called "conching", aims to improve the flavours and reduce the amount of volatile substances that negatively influence the flavour (MsShea et al. 2008). An alternative step to conching is "dutching" or "alkalinisation", a popular method that processes cocoa with alkali. This method aims to improve flavours and taste by reducing bitterness, and imparts darkness to the cocoa ingredients (Miller et al. 2008). As for cocoa, a method following alkalinisation called "fat pressing" aims to separate cocoa butter from cocoa solids, resulting in a defatted cacao product (Sivasankar 2002). Chocolate is then moulded into different shapes constituting the final product (McShea et al. 2008).

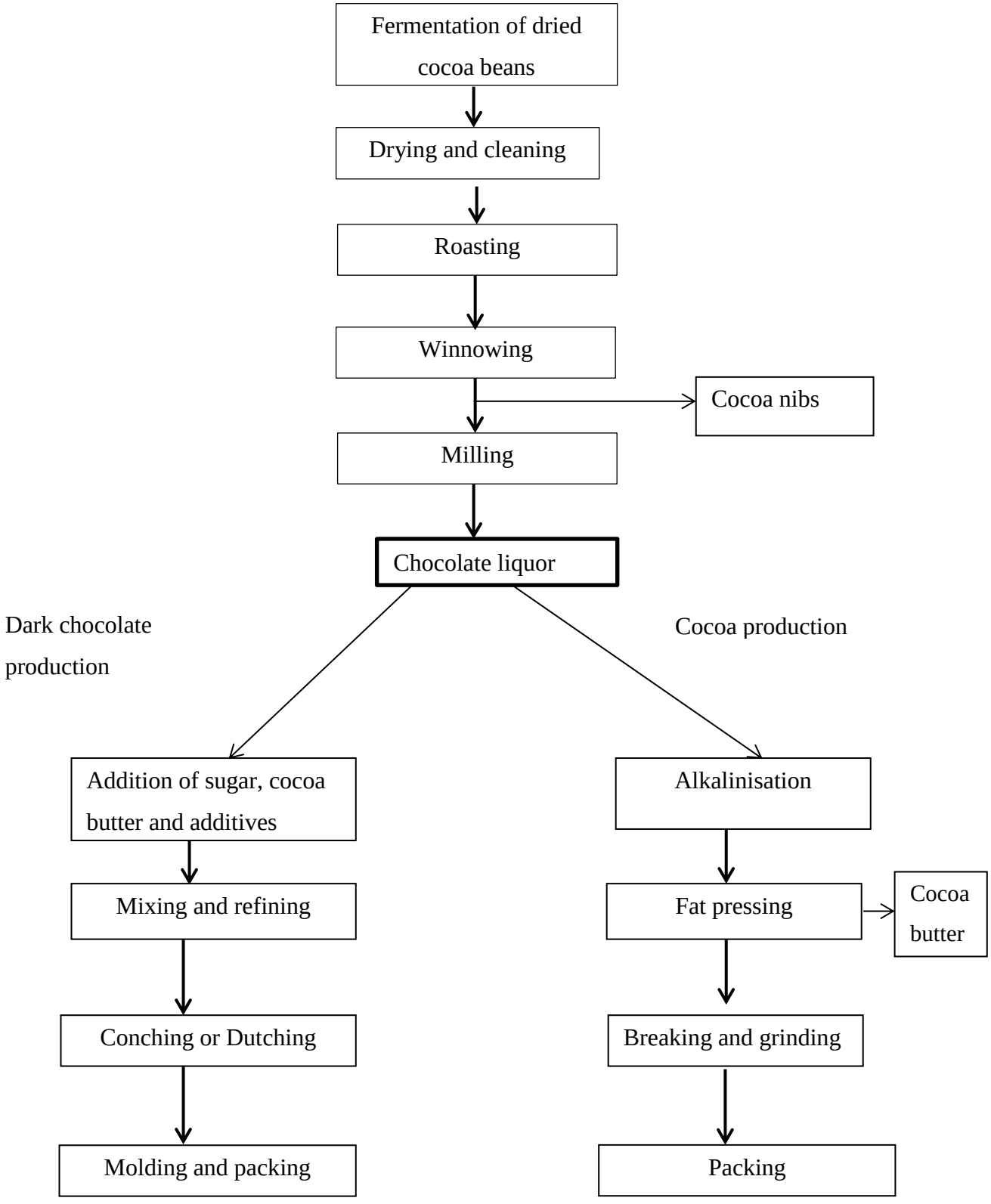



**Figure 1.16: Processing of cocoa and dark chocolate**

### 1.3.4 Effect of processing on polyphenol content

Processing of cocoa beans causes a considerable loss of polyphenols, and varies depending on the degree of processing. It has been postulated that a high temperature and an elevated pH lead to a significant loss in flavanols, particularly the monomeric ones (epicatechin and catechin). Initially, the method of drying slightly affects flavanol concentration (freeze-dried beans contain 3% more epicatechin than sun-dried beans) (Payne et al. 2010). However, fermentation, a step used in most of the processing industries, causes a significant loss of flavanols so that marginally fermented cocoa contains more antioxidants (Counet et al. 2004). The amount of flavanols decreases with the increase of the duration of fermentation, which could vary between 2-7 days. Highly fermented cocoa beans can lose up to 86% of their epicatechin content (Payne et al. 2010).

Roasting causes a significant loss of polyphenols depending on the temperature used. Losses begin to be considerable after 70$^{o}$C, and maximum losses are reached at 120$^{o}$C (losses up to 82% of epicatechin) (Payne et al. 2010). However, a study indicated that the loss of polyphenols, and consequently the reduction in the antioxidant capacity, may be restored during heat treatment by the generation of compounds during Maillard reaction such as 3-deoxyhexosuloses; the latter have been suggested to have a potential antioxidant capacity (Oliviero et al. 2008).

Dutching also causes a considerable loss of flavanols, and depends on the level of dutching. For instance, heavy dutching results in a loss of 81% of procyanidins and 78% of total flavanols (Miller et al. 2008), so that highly alkalized chocolates may only retain 2-18% of their epicatechin levels (Payne et al. 2010). In addition, dutching causes a significant decrease in quercetin and its glycosides (losses range between 58 – 86%) (Andres-Lacueva et al. 2008).

Furthermore, it has been stated that exposing the cocoa beans to high temperatures (as in roasting and alkalinization) may result in the modification of the chirality properties of catechin monomers. In fact, the abundance of (+)- catechin and –(-) epicatechin will be reduced due to the formation of –(-) catechin resulting from the epimerization of -(-) epicatechin (Kofink et al. 2007). The latter epicatechin is known to have a lower bioavailability than -(-) epicatechin (Donovan et al. 2006) (Table 1.4).

Thus, the change in the amount and properties of these flavonoids due to processing will affect the bioavailability, and consequently the potential benefits of these components (Donovan et al. 2006).

As a result, highlighting polyphenol losses at each step is vital to develop methods that aim to minimize losses and increase chocolate health benefits in the market. For instance, a recently developed processing method called “Patented Acticoa process” has been used in a large worldwide chocolate brand factory (Bisson et al. 2008). This method naturally preserves the flavanols in a way that every step of the manufacturing chain from bean to chocolate has been optimized for this purpose. For example, this method checks the ways of harvesting and treating cocoa beans (Barry Callebaut 2010), and uses non-roasted beans (Bisson et al. 2008). However, little has been said about the ways of preserving flavanols during the subsequent steps of the manufacturing process.

It is worth mentioning that there is no association between the darkness of the chocolate and the flavanol content, as it is the manufacturing of the chocolate that mainly determines its polyphenol content (Hollenberg and Ficher 2007). For instance, a highly alkalized cocoa is manufactured to a very dark chocolate that contains low levels of flavanols. Thus, a chocolate with 100% of cocoa solids may contain less flavonoid than a chocolate with 63% of cocoa solids (Langer et al. 2011).

Consequently, the amount of polyphenols largely depends on the methods of processing, and therefore the benefits of DC cannot be attributed to all the types of DC in the market.

**Table 1.4: Changes in the ratios of (-)-catechin (-)-epicatechin following roasting and alkalinisation in different cocoa samples**

| Sample | (-)-Catechin/(-)-Epicatechin |
|---|---|
| Raw, unfermented cocoa beans | - * |
| Roasted cocoa beans | 18 : 82 |
| Roasted cocoa beans (nibs) | 19 : 81 |
| Cocoa liquor (non-alkalized) | 18 : 82 |
| Chocolate (85% cocoa solids) | 18 : 82 |
| Cocoa Powder (non-alkalized) | 24 : 76 |
| Cocoa Powder (non- alkalized) | 35 : 65 |
| Cocoa Powder (alkalized)** | 42 : 58 |
| Cocoa Powder (alkalized) ** | 65 : 35 |

**Undetectable levels of –(-) catechin*
***Grade of alkalization not known*
(Kofink et al. 2007)

### 1.3.5 Bioavailability of cocoa polyphenols in the human body

Because of their complexity, the bioavailability of cocoa polyphenols is not completely understood. This has affected the understanding of the health benefits of these components. As they are stable in an acidic environment, cocoa flavanols escape the digestion in the stomach, and reach the small intestine intact. In the small intestine, flavanols rapidly undergo glucuronidation and methylation in order to be absorbed. The fact that plasma concentrations of cocoa polyphenols are in the micromolar and nanomolar range following ingestion may have several explanations, such as the conversion of these polyphenols into metabolites and the rapid metabolism and elimination of polyphenols. This makes it difficult to detect these metabolites in the blood (Ali et al. 2014).

-(-)Epicatechin and (+)-catechin are considered highly bioavailable due to their chirality properties (Cooper et al. 2008), and are extensively metabolized and readily adsorbed into the bloodstream, with epicatechin having the highest bioavailability (McShea et al. 2008). However, as previously stated, due to cocoa processing, some of the –(-)epicatechin portion may be epimerized to –(-catechin) (Kofenk et al.

2007), which has been reported to have a poor bioavailability and a lower intestinal absorption than –(-) epicatechin (Donovan et al. 2006). This will most likely affect the beneficial properties of cocoa containing products. The metabolites (such as 3'-O-methylepicatechin) and the non methylated epicatechins (such as epicatechin-7-sulfate) reach the blood an hour after ingestion, in the micromolar range and in a dose dependent manner. The peak plasma concentration of metabolites occurs 2.5 hours after flavanol-rich foods consumption and reaches baseline levels after approximately 8 hours (Hackman et al. 2008). Regarding procyanidins, a small part of procyanidin dimers is metabolized and absorbed in the small intestine (Rios et al. 2003) (the same way as catechin and epicatechin), and then undergo conjugation, sulfation and glucuronidation in the liver (keen et al. 2005). Procyanidin dimer B2 has been detected in human plasma following the consumption of a cocoa drink (Holt et al. 2002). However, procyanidins with a higher degree of polymerization are not absorbed in the small intestine (absorption of procyanidins with a degree of polymerization > 5 is estimated to be 1%), and are then fermented by the colonic microflora, leading to phenols with low molecular weight (Holt et al. 2002; Manach et al. 2005) such as vanillic acid and ferulic acid (Rios et al. 2003). Although it was reported in studies with rats that a small fraction of epicatechin in plasma can result from the breakdown of cocoa procyanidins (Baba et al. 2002), another study suggests that the degradation of procyanidins is not possible in humans. This was explained by the need for a higher degree of acidity to hydrolyse procyanidins in the human stomach (Rios et al. 2002). Consequently, the poor intestinal absorption of procyanidins may suggest that they exert their effects locally in the gastrointestinal tract, or through phenolic acids generated during their degradation in the colonic microflora (Manach et al. 2004).

As for quercetin, its bioavailability in cocoa has not been extensively studied, and the exact metabolites known to reach the plasma following cocoa consumption have not been clearly elucidated. Yet, it has been documented that quercetin glucosides (the most prevalent forms of quercetin in cocoa) are absorbed via the small intestine, and some quercetin metabolites have been identified in the colon (Manach et al. 2005). Further studies looking at the bioavalibility of quercetin in human plasma following cocoa/DC consumption are then needed. Interestingly, a study showed that

epicatechin and quercetin can even cross the blood brain barrier through unclear mechanisms (Abd el Mohsen et al. 2002), which might explain the possible implication of cocoa/DC in neurodegenerative disorders.

Furthermore, other factors may affect the bioavailability of cocoa polyphenols: A meal high in carbohydrates (bread or sugar) could increase the absorption of flavanols in a sugar free cocoa-rich flavanols by 140 % compared to consuming sugar free cocoa-rich flavanols alone. However, lipids and proteins seem to minimally affect the absorption of these flavanols. Many explanations have been provided regarding this effect. Carbohydrates may influence the physiology of the gastrointestinal tract including motility and secretion, which enhances the uptake of flavanols. An implication of carbohydrate-flavanol transporter that enhances flavanol absorption has also been suggested. Yet, this transporter has not been yet identified (Schramm et al. 2003). In addition, the consumption of milk with cocoa product has been controversial: while a study noted an impact of milk on decreasing the bioavailability of cocoa polyphenols (Serafini et al. 2003), other studies have not showed such influence (Keogh et al. 2007; Roura et al. 2007).

### 1.3.6 Benefits of cocoa/dark chocolate

In the last decade, many studies have highlighted the effects of cocoa/chocolate on health. However, the conflicting results and the huge amount of marketing and scientific information rendered the consumer undetermined whether the chocolate is beneficial or detrimental to health (McShea et al. 2008). In a recent statement of the European food safety authority (EFSA), a relationship between cocoa flavanols and the maintenance of a healthy endothelium has been claimed. This suggests a beneficial effect of these flavanols on the prevention CVD in the general population (EFSA 2012).

Studies on cocoa/dark chocolate have primarily investigated their effects on risk factors for type 2 diabetes and CVD, including lipid profile, antioxidant capacity, insulin resistance, blood pressure and inflammation, suggesting a possible implication of cocoa/DC in tackling the metabolic syndrome (These effects will be discussed in detail in sections 1.4 - 1.10).

Cocoa has been proposed to have beneficial effects on visual functions and cognitive performance (which are explained by the increased cerebral blood flow observed following cocoa flavanol consumption) (Field et al. 2011), and immune system (due to the effect of cocoa on increasing the percentage of B-cells (Ramiro-puig et al. 2009). Additionally, cocoa has been claimed to have anti-cancer properties (due its antioxidant properties) (Carnésecchi et al. 2002), skin protection properties against oxidative damage (because of its ability to increase dermal flow) (Neukam et al. 2007), as well as a neuroprotection role against inflammation and neurodegradation, by preventing death of neurons (Joseph et al. 1999). This may confer a source of protection against neuroinflammatory diseases like Parkinson and Alzheimer diseases (Francis et al. 2006). However, most of these studies have been carried out on animals or in vitro, highlighting the need for human studies to evidence these effects.

Nonetheless, despite all the favourable effects of dark chocolate on health, there has been some discrepancy over the consumption of dark chocolate, suggesting that the beneficial effects of cocoa may be limited to its raw consumption, the way it was previously consumed by the primary civilizations. Additionally, DC may contain a high amount of saturated fat, sugar and added vegetable oils (McShea et al. 2011), which increase energy intake in unbalanced diets, and can lead to weight gain (Rimbach et al. 2011). Moreover, cocoa and chocolate were associated with significantly higher pulse rates in older adults. However, this has been attributed to the methylxantine content of DC (theobromine and caffeine), known to exert a stimulant effect (Crews et al. 2008).
Given the potential adverse outcomes and the reported beneficial effects of chocolate on human health in a large number of research studies, it is important to weigh the benefits of chocolate against the unhealthy components, and consume it in moderate amounts (McShea et al. 2011) to avoid its potential undesirable effects.

### 1.3.7 Difference between cocoa and dark chocolate

Although the terms “cocoa” and “dark chocolate” often overlap in the literature, these two products differ by polyphenol and fat composition. Generally, cocoa

powder contains a higher amount of total polyphenols compared to DC (Miller et al. 2004), as well as a higher amount of catechin and epicatechin (Natsume et al. 2000), and consequently a higher antioxidant capacity (Miller et al. 2006). Furthermore, due to fat pressing (Figure 1.16), cocoa contains less cocoa butter and consequently a lesser amount of fat than DC (Sivasankar 2002) (Table 1.5 provides examples of polyphenol and fat contents of commercial cocoa and DC brands). These differences in fat and polyphenol content could make the comparison between cocoa and DC inaccurate, particularly when studying their effect on body weight and lipid metabolism. This has led to the suggestion that the recent beneficial effects of cocoa polyphenols on reducing obesity cannot be attributed to ordinary chocolate products (Ali et al. 2014). Ideally, studies on cocoa should only provide a background for carrying out human investigations looking at the effects of DC. However, since the quantity of polyphenols was controlled by the companies that provided cocoa or chocolate to research studies, the polyphenol amount was not necessarily higher in cocoa compared to chocolate. Furthermore, despite the difference in fat ratio, the energy content of the daily dose of DC was not commonly greater than the energy provided by cocoa in many research studies that investigated the effect of cocoa/DC on cardiovascular health (view Table 1.6 on examples of the fat and polyphenol content of cocoa/DC used in research studies). In addition, studies have previously shown that the total energy of a diet is more effective than its fat content in reducing weight, suggesting that calorie counts more than fat. This was demonstrated in weight loss studies, where a low calorie diet and a low fat/low calorie diet were equally effective in reducing weight (Harvey-Berino 1999; Djuric et al. 2002). Moreover, it seems there are no significant differences in the plasma or urine concentrations of flavanols after the consumption of the same amount of flavanols from chocolate or dry cocoa (Baba et al. 2000). Consequently, acknowledging the energy content and the amount of polyphenols of cocoa and dark chocolate in research studies will provide a better way of comparison between the two cocoa tree derivatives.

Nevertheless, a problem remains in extrapolating the results of cocoa studies to chocolate, and making recommendations to the general population through commercial cocoa and chocolate products. The difference in the amounts of

polyphenols and energy between the two products may possibly affect the properties of DC.

**Table 1.5: Polyphenol and fat content of selected commercial cocoa and dark chocolate products**

| Product | Fat percentage % | *Total polyphenol content (mg/g) | Epicatechin +catechin content (mg/g) |
|---|---|---|---|
| Cocoa powder 1 | 12.4 | 60.2 | 3.723 |
| Cocoa powder 2 | 21.7 | 45.3 | 1.610 |
| Cocoa powder | 11 | 51.7 | 1.963 |
| DC-1 | 33.4 | 11.7 | 0.478 |
| DC-2 | 40.7 | 14.9 | 0.149 |
| DC-3 | 30 | 12.3 | 0.605 |

(Adapted from Miller et al. 2009)
**Polyphenol content is expressed as mg of gallic acid equivalents (GAE)*
*DC: Dark chocolate*

**Table 1.6: Examples of studies showing the difference in energy and polyphenol contents between cocoa and dark chocolate**

| Study | Cacao derivative used/ daily amount | Amount of polyphenols (mg/g) | Amount of Fat (g) | Total Energy (Kcal) |
|---|---|---|---|---|
| Allen et al. (2008) | DC/ 22g | 360 | 12 | 200 |
| Al Moosawi et al. (2012) | DC/20g | 500 | 7.34 | 108 |
| Baba et al. (2007b) | Cocoa /26g | 234 | 2.99 | 122 |
| Monagas et al. (2009) | Cocoa/40g | 495.2 | 2.1 | 136 |

*DC: Dark chocolate. Amounts of cocoa/DC represent the daily doses used in research studies.*

## 1.4 Effects of cocoa/dark chocolate on inflammation and antioxidant capacity

As mentioned in section 1.1, oxidative stress and chronic inflammation are both considered playing a pivotal role in the occurrence of CVD (Urquiaga and Leighton 2000; Shah 2009). In fact, a NO-dependent link has been suggested between oxidative stress, inflammation and cardiovascular risk (Rein et al. 2000) (Figure 1.17). Additionally, oxidative stress can possibly lead to the activation of NF-κB which stimulates the inflammatory cascade, and may result in the production of the inflammatory C-reactive protein (CRP) (Wanner and Metzger 2002).

Several epidemiological studies have found an inverse association between regular chocolate consumption and CVD (Djousse et al. 2001; Buijsse et al. 2010; Mostofsky et al. 2010). In a meta-analysis of cross-sectional and cohort studies, highest levels of chocolate consumption have been linked to a lower risk of CVD (by 37%), and a lower risk of stroke (by 29%) (Buitrago-Lopez et al. 2011). Several mechanisms have been suggested, yet there has been no agreement on the role of cocoa polyphenols in CVD. The preventive effect of cocoa/DC on CVD has been postulated to be due to four main properties of flavonoids: anti-inflammatory, anti-oxidant, anti-platelet aggregation and vasodilative properties (Pucciarelli 2013). This section will mainly focus on the antioxidant and anti-inflammatory functions of cocoa polyphenols.

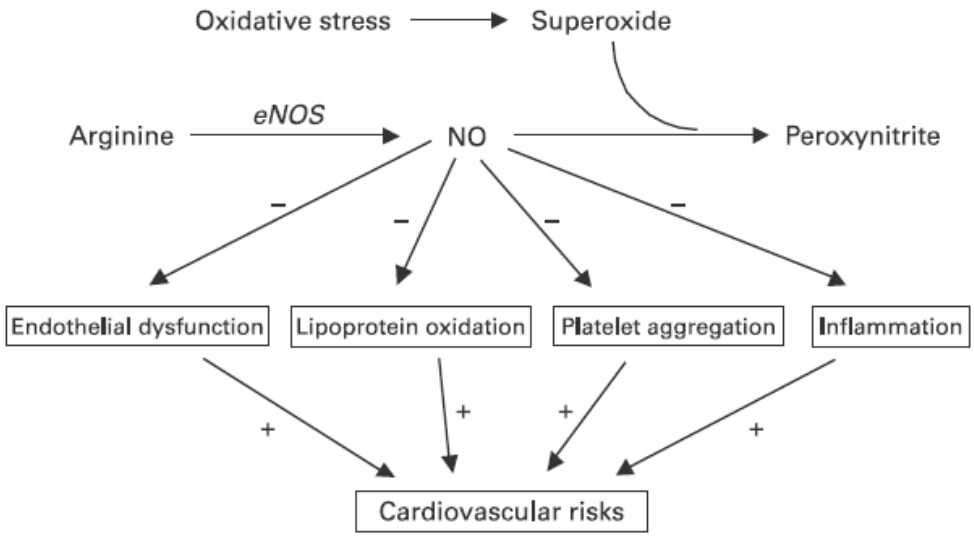



**Figure 1.17: Nitric oxide-dependent association between oxidative stress, inflammation and cardiovascular risks**

(Cooper et al. 2008)
*NO: Nitric oxide, eNOS: Endothelial nitric oxide synthase. NO exerts an inhibitory effect on inflammation, oxidation and endothelial dysfunction, which can increase cardiovascular risk.*

The antioxidant properties of flavonoids in cocoa/DC have been mainly related to their chemical structure (Galleano et al. 2009). The aromatic ring (specifically the catechol group on the B ring (Sekher Pannala et al. 2001)) allows flavonoids to act as scavengers for ROS. Flavonoids also participate in the chelation of metals that enhance ROS (Cotelle 2001), and in the inhibition of enzymes like xanthine oxidase and NADPH-oxidase (and consequently the inhibition of the reaction between $O_2$ generated by these enzymes and NO). This leads to an increase in NO and to the protection of the endothelium (Engler and Engler 2006) (Figure 1.18). The increase in NO production by flavanoids has been also suggested to be due to a mechanism involving the decrease in the activity of vascular arginase, which competes with the enzyme iNOS for L-arginine (Schnorr et al. 2008). Also, cocoa flavanols interact with the NF-Kb pathway, resulting in a decrease in its activation (Mackenzie et al. 2002). Furthermore, the hydrophobic and hydrophilic ends in flavonoids allow the latter to interact with the phospholipids on the membrane surfaces of lipids, proteins and nucleic acids, and modify their membrane properties (Oteiza et al. 2005),

leading to their protection from oxidative damage (Rein et al. 2000). Enzymes like lipoxygenase and COX-2 can also be inhibited by cocoa flavanols (Galleano et al. 2010). Lastly, the antioxidant capacity of polyphenols has been explained by the fact that PPs increase the gene and protein expression of antioxidant enzymes like superoxide dismutase (SOD) and glutathione oxidase (Rahman et al. 2006).

Several markers of oxidation have been studied. In human studies lasting 2 - 4 weeks, cocoa/DC supplementation has been effective in increasing flow mediated dilation (FMD) (an indicator of endothelial dysfunction) (Hooper et al. 2008), ascorbic acid (Murphy et al. 2003), TBARs (Thiobarbituric acid reactive substances) (Wan et al. 2000), oxygen radical absorbance capacity (ORAC) (Al Moosawi et al. 2012), F2-isoprostanes (Lettieri-Barbato et al. 2012), and decrease vascular constriction of the brachial artery (West et al. 2014). Cocoa polyphenol extract has been recently shown to reduce peroxynitrite, along with an increase in NO levels in men and women (Nanetti et al. 2013).

Regarding LDL oxidation, which has been shown to be a better predictor of coronary artery diseases than lipid levels (TG, HDL and LDL) (Ehara et al. 2001), results have been promising. Previous research showed that in vitro, cocoa and cocoa flavonoids prevent the oxidation of LDL (Salah et al. 1995; Kondo et al. 1996) in a greater extent than red wine polyphenols (Waterhouse et al. 1996). Cocoa polyphenols have also inhibited LDL oxidation in rabbits with hypercholesterolemia (Kurosawa et al. 2005), and in rats fed a high fat diet (Osakabe and Shibata 2012). These findings have been supported by several human studies. A crossover study demonstrated that LDL oxidation is decreased after 4 weeks of consuming a diet enriched in cocoa and dark chocolate (containing 466 mg of procyanidins) by 23 healthy individuals. This was evidenced by a longer lag time (an indicator of oxidative status, and is defined as the time required for the consumption of an antioxidant) which increased by 8% ($p= 0.03$) in this group. However, there was no significant change in LDL oxidation following the consumption of an American diet free of DC and cocoa. This decrease in LDL oxidation was accompanied by an increase in HDL ($p= 0.02$) (Wan el al. 2001), suggesting an inverse association between the two parameters. Baba et al. (2007b) demonstrated that cocoa consumption (containing 174 mg of flavanols)

decreased LDL oxidation in 25 healthy subjects, indicated by a 9.4% increase in lag time after 12 weeks ($p<0.001$). A significant decrease in lag time (19.8%, $p< 0.001$) was noted in participants in the control group. LDL oxidation levels were also decreased after 4 weeks of the consumption of cocoa high in polyphenols (140-281 mg) in normocholesterolemic and hypercholesterolemic participants ($p<0.05$) (Baba et al. 2007a). Moreover, a study performed on 15 volunteers showed that the consumption of cocoa powder rich in polyphenols for 2 weeks (2610 mg) decreased LDL oxidation susceptibility by increasing the lag time of LDL oxidation (Osakabe et al. 2001). An increase in lag time of LDL oxidation by 9.8% ($p< 0.05$) was also noted after 6 weeks of the daily consumption of cocoa and dark chocolate rich in polyphenols (651 mg) (Mathur et al. 2002). Lastly, the consumption of ready-to-eat meals supplemented with cocoa extract (containing 645 mg of polyphenols) within a hypocaloric diet resulted in a greater reduction in plasma oxidised LDL ($p= 0.03$) compared to a hypocaloric control diet with no cocoa extract (Ibero-Baraibar et al. 2014). Although a study conducted on 21 healthy adults failed to show a significant change in LDL oxidation susceptibility when participants were assigned either a high flavonoid dark chocolate (259 mg of flavonoids) or a low flavonoid chocolate for 2 weeks (Engler et al. 2004), most of the studies in the literature support a beneficial effect of DC or cocoa on LDL oxidation. This effect has been mainly explained by the binding of flavonoids to the surface of LDL particles, leading to an increase in the resistance of LDL to oxidation, by either chelating metals or scavenging oxygen radicals (Terao 1999).

The antioxidant effect of cocoa polyphenols has been mainly attributed to the monomeric fraction of flavanols. A meta-analysis of seventy human studies on cocoa showed that epicatechin increases NO synthesis via inhibition of NADPH oxidase and arginase (Ellam and Williamson 2013), and protects against oxidative stress induced by oxidised LDL (Schewe et al. 2008). Epicatechin was effective in protecting LDL and VLDL from oxidation when the latter particles were isolated from the plasma of normocholesterolemic individuals. This was demonstrated by a longer lag time in cells administered epicatechin (Vinson et al. 1995). The enantiomer –(-)epicatechin and its metabolite epicatechin-7-O-glucuronide seemed

to exert the most prominent role on the increase in the synthesis of NO (Schroeter et al. 2006).

Procyanidins were also implicated in increasing the lag time of LDL oxidation (Pearson et al. 2001), lowering DNA oxidative damage (Ottaviani et al. 2002), and attenuating the transcription of NF-kB (Mackenzie et al. 2004). Cocoa procyanidins also inhibited LDL oxidation in vitro (Mazur et al. 1999). Moreover, quercetin was demonstrated to have an inhibitory role on LDL oxidation in vitro via the modulation of pathways involved in oxidative damage (Choi et al. 2009). Yet, it is not clear whether the cocoa content of quercetin is sufficient to induce such effect. These findings lead to the conclusion that the different fractions of cocoa polyphenols may exert a combined effect on antioxidant capacity. Further human studies are needed to elucidate this effect.

However, there have been some doubts regarding the antioxidant capacity of cocoa polyphenols. This is mainly due to the low plasma polyphenol concentration achieved after the consumption of foods rich in flavonoids (estimated to be in the micromolar and nanomolar range) (Spencer et al. 2001; Holt et al. 2002), mainly due to the low bioavailability of polyphenols in humans (Lotito and Frei 2006). In fact, the administration of a single dose of radiolabeled flavonoids to rats showed that these polyphenols were mainly traced in stomach, intestine and liver with very low concentrations reaching the bloodstream (Mullen et al. 2002). This raises questions over the ability of these flavonoids (and mainly flavanols) to exert free radical scavenging properties, particularly when present in the blood simultaneously with other antioxidant compounds like tocopherols with much higher concentrations (Holt et al. 2002). In addition, the accumulation of polyphenols in tissues is not well elucidated (Manach et al. 2004). Hence, the antioxidant capacity of cocoa flavanols may be restricted to the blood and gastrointestinal tract, which are exposed to flavanol doses right after consumption (Fraga 2007). Further research using radiolabelled flavanols is needed to identify their mechanism of action as antioxidants in humans.

In view of this, it is possible that polyphenols metabolites that are generated by the colonic microflora (which most of them are still unknown and their concentration can reach hundreds of micromoles/liter in the colon) are responsible for exerting most of the polyphenols benefits, including antioxidant properties. For example, the administration of radiolabelled quercetin ([2-14C]quercetin- 4'-O-glucoside) showed that the biggest fraction of quercetin reached the colon and was degraded into benzoic acid and 3-hydroxyphenylacetic acid, while there was no accumulation of this flavonoid in tissues (Mullen et al. 2002). Benzoic acid has been previously regarded to have a potential antioxidant capacity (Manach et al. 2004). Rios et al. (2003) showed that after cocoa consumption, the colonic metabolites 3-(3'-hydroxyphenyl) propionic acid, 3'hydroxyphenyl acetic acid, 3-hydroxybenzoic acid and ferulic acid were present in the urine. Other colonic metabolites such as 5-(3',4'-methoxy-4'-hydroxyphenyl)- γ-valerolactone and 5-(3',4'-dihydroxyphenyl)- γ-valerolactone were found in the urine after administration of cocoa powder (Urpi-Sarda et al. 2009). In particular, the 5-(3',4'-dihydroxyphenyl)- γ-valerolactone metabolite produced from the hydrolysis of +(-) catechin in the colon was shown to have a higher antioxidant capacity compared to +(-) catechin. This metabolite was also shown to inhibit metalloproteinases and therefore decrease inflammation (Monagas et al. 2010). Furthermore, the metabolite 3,4-dihydroxytoluene (a metabolite of quercetin) derived from gut microbiota was demonstrated to inhibit lipid peroxidation in the hepatocytes of rats (Glässer et al. 2002). Research is still limited regarding the role and the mechanisms of action of polyphenol metabolites produced in the colonic microflora on health.

An indirect mechanism on antioxidant capacity via the upregulation of antioxidant enzymes (such as SOD) by cocoa polyphenols has been suggested in humans (Rahman et al. 2006). Further research looking at the mechanism involved in the antioxidant role of cocoa polyphenols in humans is needed to support these effects.

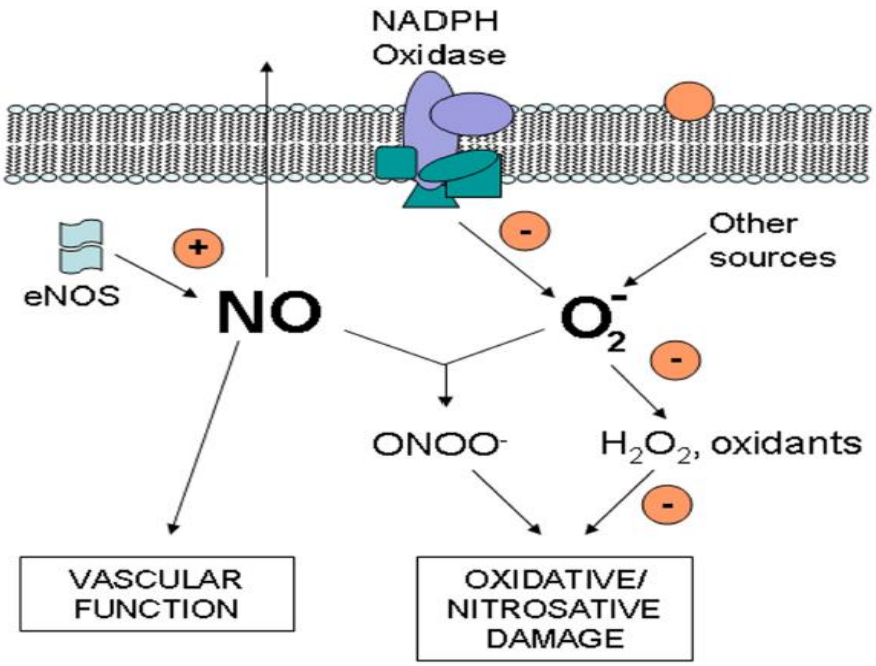



**Figure 1.18: Mechanisms by which flavanols can possibly reduce oxidative damage**

(Galleano et al. 2009)
*NO: Nitric oxide, eNOS: endothelium nitric oxide synthase, $O2^-$: Superoxide, NADPH: Nicotinamide adenine dinucleotide phosphate hydrogenase, $ONOO^-$: peroxynitrite, $H_2O_2$: hydrogen peroxide. Flavanols (indicated by circles) inhibit NADPH oxidase (which generates $O_2^-$ and leads to the production of Reactive oxygen species) and increase NO production through the activation of eNOS. Flavanols can act by modifying membrane properties leading to an increase in NO and superoxide production. NO will attain smooth muscle cells and lead to vascular relaxation.*

Regarding the effects of cocoa/DC on inflammatory markers, results are still controversial. Most of the favourable effects on inflammation have been reported by "in vitro" studies. Cocoa flavonoids have been shown to decrease the production of COX-2, as well as pro-inflammatory cytokines (like Interleukin 2 (IL-2) and prostaglandins (Selmi et al. 2006). Cocoa polyphenols decreased inflammation by modulating the activity of eicosanoids (vasoconstrictor), through the decrease of leukotrienes and the increase of prostacyclin (a vasodilator) (Schramm et al. 2001), and the inhibition of the synthesis and gene expression of cytokines (Middleton et al. 2000). The potential effect of cocoa polyphenols on inflammation has been attributed to flavanols (particularly epicatechin) which inhibit ROS (Corti et al. 2009), and interfere with the NF-KB pathway. Furthermore, in vitro studies showed that cocoa procyanidins inhibited metalloproteinase in human aortic cells (Lee et al. 2008).

Among inflammatory markers, CRP has a particular importance, as apart from being a biomarker of chronic inflammation as well as a marker of CVD (Chun et al. 2008), it is also a contributor to endothelial dysfunction (Pasceri et al. 2000). Indeed, CRP may participate in the uptake of LDL by macrophages and the formation of foam cells, thus helping in the initiation of the atherosclerotic plaque (Zwaka et al. 2001). CRP may also increase tissue damage in the event of plaque rupture (Zebrack and Anderson 2002).

High-sensitivity CRP (hs-CRP) has been shown to be a stronger predictor of coronary artery diseases than CRP (Jousilahti et al. 2001): A high hs-CRP has been correlated with 3-4 fold higher risk of myocardial infarction in men (Ridker et al. 1997). Hs-CRP is considered the test of choice when assessing inflammation in individuals with no history of CVD. The reason is due to the inaccuracy of CRP test in detecting low CRP levels in healthy individuals (Zebrack and Anderson 2002). Hs-CRP can be helpful in the primary prevention of CVD, as it helps to detect early signs of coronary risk (Ridker 2001). Hs-CRP is principally stimulated by IL-6, a predictor of coronary diseases. Levels of hs-CRP have been reported to be higher with increasing BMI, suggesting a low grade inflammation state in the obese category (Visser et al. 1999).

Studies investigating the effect of cocoa polyphenols on CRP and hs-CRP have led to confounding results: Epidemiological studies have mainly documented positive findings. Data from NHANES (National Health and Nutrition Examination Survey) 1999-2002 have been used for a cross-sectional study, and showed that dietary flavonoids are inversely correlated with serum CRP levels ($p<0.05$) in 8335 adults (Chun et al. 2008). In addition, a cohort study showed that after adjustment of many confounding factors such as age, sex, social status, BMI and energy intake, regular dark chocolate eaters have lower hs-CRP levels ($p= 0.038$) than subjects who do not regularly eat chocolate. Interestingly, hs-CRP levels were lower in moderate consumers (20g of DC every 3 days) than higher consumers (20g of DC/day) (Gisueppe et al. 2008), suggesting that a moderate amount of chocolate is more beneficial on cardiovascular risk than higher amounts.

On the other hand, intervention studies have been less likely to show a favourable effect of cocoa/DC on hs-CRP/CRP levels. Hamed et al. (2008) reported a decrease in hs-CRP only in women (from 1.8± 2.1 mg/l to 1.4±1.7 mg/l, $p< 0.04$) after one week of daily DC supplementation (providing 700 mg of flavonoids), and Stote et el. (2012) indicated a decrease in CRP levels following 5 days of cocoa drink supplementation (containing doses of PPs ranging from 30-900mg) in a dose dependent manner ($p = 0.01$) (by 0.7 mg/l with the highest polyphenol dose). However, the decrease in hs-CRP/CRP levels was small in the latter two studies, thus it is unlikely that these changes are clinically relevant. Most of other studies failed to demonstrate this. Grassi et al. (2008) showed no significant effect on CRP levels after 2 weeks of flavonoid-rich chocolate (500 mg) supplementation in hypertensives. Similar results were noted in healthy individuals. Mathur et al. (2002) did not note a significant effect of 6 weeks of cocoa supplementation (651mg of procyanidins) on the markers of inflammation including hs-CRP, IL-1, IL-6 and TNF-α. This outcome was explained by the short biological half-life of flavonoids in cocoa products, resulting in a decrease in their plasma levels shortly after supplementation, and making flavonoids less likely to exert an effect on inflammation (Mathur et al. 2002). In addition, a recent study showed no effect on the inflammatory markers TNF-α, IL-1 α, IL-1β, IL-6 and hs-CRP after 7 days of DC supplementation (with 2000 mg of polyphenols) (Di Renzo et al. 2013). Also, hs-CRP levels were not significantly changed after the intake of DC rich in polyphenols (180 mg) for 4 weeks (Allen et al. 2008), and Nogueira et al. (2012) showed no significant changes in hs-CRP levels after four weeks of high polyphenol chocolate consumption (2135 mg).

Furthermore, a meta-analysis of 5 studies found no significant impact of flavonoid rich chocolate on circulating CRP levels, a finding that remained constant after subgroup analysis based on baseline health status (Shrime et al. 2011). These results were consistent with another meta-analysis of 10 studies, which did not show a significant effect of cocoa/DC on CRP levels (Hooper et al. 2012).

These controversial results may suggest an adaptation effect to the quantity of flavonoids, which explained the positive results observed in some of the short-term studies (Hamed et al. 2008; Stote et al. 2012). Further studies are needed to clarify

the discrepancy between epidemiological and intervention studies when studying the effect of cocoa/DC on inflammation. However, as epidemiological studies do not show cause and effect (CDC 2004), only associations have been made. In addition, this discrepancy may be due to other confounding factors that have not been controlled in epidemiological studies. A prominent example is the proportion of saturated fatty acids in the diet. The latter factor is known to increase inflammation (Chait and Kim 2010), and may have confounded the relationship between frequent chocolate eaters and CRP.

## 1.5 Effects of cocoa/dark chocolate on insulin and glucose metabolism

There has been a considerable amount of research on polyphenols and their effect on biomarkers of glucose metabolism. For instance, IR and glucose levels have been reported to decrease following the administration of resveratrol in mice (Chen et al. 2012) and EGCG in rodents (Bose et al. 2008). Flavonoid-rich berries extracts have decreased intestinal uptake of glucose in human intestinal cells (Alzaid et al. 2011). These findings provide a growing body of evidence that polyphenols may be effective in improving glucose and insulin metabolism in humans, and helping in the management/prevention of diabetes. Nevertheless, human data on cocoa polyphenols and insulin and glucose metabolism remain relatively limited. This is mainly due to the limited animal studies and the lack of knowledge of the mechanisms of action of cocoa flavanols (Dorenkott et al. 2014). In fact, although few studies have proposed a preventive effect of cocoa polyphenols on IR and glucose levels, the mechanisms responsible for these effects remain unclear. Additionally, these mechanisms have been mostly suggested through research on animal and in vitro (View Figure 1.19 on the mechanisms of action of cocoa polyphenols) and have not been yet evidenced by studies on humans.

Cocoa phenolic extracts have been documented to inhibit alpha-amylase (Quesada et al. 1996), to reduce the postprandial elevation of glucose in rats (Tanaka et al. 2004), and to decrease hyperglycemia in diabetic rats (Song et al. 2002). Furthermore, the effect of cocoa polyphenols on insulin sensitivity has been proposed to be due to the

link between insulin sensitivity and NO bioavailability in endothelial cells (Konopatskaya et al. 2003; Faridi et al. 2008). IR has been reported to be a cause or a consequence of endothelial dysfunction (Sydow et al. 2005). In fact, by increasing the bioavailability of NO, cocoa flavanols enhance vasodilatation, and result in improving insulin-stimulated blood flow, which increases the supply of insulin to target tissues like skeletal muscle and liver (Sydow et al. 2005). In humans, this was supported by the decrease in IR along with an improvement of endothelial function (Grassi et al. 2005; Kim et al. 2006), and the simultaneous decrease in blood pressure and IR in the 2 studies conducted by Grassi et al. (2005; 2008), suggesting a link between insulin sensitivity, blood pressure and endothelial dysfunction. NO has been also suggested to stimulate GLUT4 leading to an increase in cellular glucose uptake (Etgen et al. 1997).

As for the fraction of polyphenols involved in the beneficial effect on insulin responses and glucose metabolism, epicatechin has been previously regarded as the main agent responsible for such effects. Epicatechin increases pancreatic β-cell insulin secretion (Chakravarthy et al. 1982; Hii et al. 1985), possibly via modulation of $Ca^{2+}$ metabolism ($Ca^{2+}$ is the key regulator in the secretion of β-cells (Xuelin et al. 2001)), and its promotion of the release of insulin (Rutter 2001). This has explained one of the mechanisms by which epicatechin may prevent the occurrence of type 1 diabetes in mice (Fu et al. 2013). Results were reinforced by a human study which showed that epicatechin decreases postprandial hyperglycemia in 20 adults (Gutiérrez-Salmeán et al. 2014). Procyanidins from phenolic extracts have also contributed to the improvement in glucose metabolism through to the inhibition of the activity of α-glucosidase, as well as salivary and pancreatic α-amylases in vitro (McDougall et al. 2005). Procyanidins have been also implicated in the decrease of the degradation of GLP-1 (Glucagon-like peptide 1), a hormone that stimulates insulin β-cell secretion and inhibits gastric emptying (Drucker 2002). Furthermore, procyanidins have been proposed to inhibit endotoxemia. Endotoxin, known as an important mediator of low-grade inflammation, and a component of the membrane of bacteria in the colon, has been related to obesity and IR. Endotoxin induces the Toll-like receptors (TLR), which activate NF-kb and stimulate inflammation leading to an

impairment of insulin signalling in skeletal muscle. Procyanidins have been effective in inhibiting TLR4 (Sung et al. 2013), constituting one of the mechanisms by which procyanidins may lower IR. These findings suggest that procyanidins exert their main function in the gut, since they have a low bioavailability and are mainly unabsorbed (Dorenkott et al. 2014). This was further demonstrated by the ability of procyanidins to improve glucose tolerance only when given orally, but not when injected into the peritoneum (Gonzalez-Abuin 2012). Lastly, in addition to its functions in the gut, procyandins have been proposed to ameliorate pancreatic β-cell function, thus playing an additional role in glucose metabolism. Yet, it is not clear whether this role is exerted via metabolites generated in the colonic flora, or by direct effect of procyanidins (Dorenkott et al. 2014). Additionally, a cell study showed that epicatechin and cocoa polyphenolic extracts are equally effective in improving insulin sensitivity in human hepatic cells (HepG2) treated with high glucose. This was demonstrated by the ability of both fractions to improve tyrosine phosphorylation and insulin receptors (including IRS-1 and IRS-2) and to activate PI3K/AKT pathway in hepatic cells. Furthermore, epicatechin and CPE (cocoa polyphenol extract) have been documented to attenuate the increase in PEPCK levels (which play a role in gluconeogenesis) (Cordero-Herrera et al. 2013).

Moreover, the flavonol quercetin has been reported to decrease hyperinsulinemia (Li et al. 2013), and to improve glucose absorption and transport through the inhibition of the glucose transporter GLUT2 (which facilitates intestinal glucose absorption) (Song et al. 2002; Chen et al. 2007), and the digestive enzymes α-amylase and α-glucosidase (both implicated in the digestion of carbohydrates) (Tadera et al. 2006) in vitro. Quercetin has also been shown to interact with the sodium-dependent glucose transporter (SGLT1) in the intestinal mucosa in vesicles of porcine jejunum, resulting in its inhibition, and to decrease in glucose uptake (Cermak et al. 2004). Again, it is not clear whether the dose of quercetin supposed to exert such effects is sufficient in chocolate.

In addition, the implication of the colonic microflora in improving insulin sensitivity and glucose metabolism following polyphenol administration can also be suggested. In fact, undigested carbohydrates (resulting from the inhibitory effect of PPs on digestive enzymes) enter the colon. They are then converted into short-chain fatty

acids (SCFA) (such as propionic and butyric acids) which are subsequently absorbed. Studies demonstrated a potential role of SCFA in improving insulin sensitivity by stimulating GLP-1 secretion; the latter has been shown to increase β-cell proliferation, to improve glucose-dependent insulin secretion and to inhibit gastric emptying (which results in the decrease in glycemic excursion following meal consumption by slowing the rate of nutrient passage into the small intestine) (Drucker 2002). However, the mechanisms linking SCFA and GLP-1 secretion remain poorly understood. Also, by reducing inflammation (through decreasing the secretion of proinflammatory cytokines), SCFA can reduce IR and decrease serum glucose levels (Puddu et al. 2014) (The role of inflammation in the occurrence of IR is discussed in section 1.1.4.4). Lastly, SCFA have been implicated in increasing satiety and decreasing food intake through several mechanisms such as the inhibition of gastric emptying via the increase of GLP-1 (Drucker 2002). Importantly, the administration of GLP-1 to rodents reduced short term and long term food intake (Szayna et al. 2000). This suggests a potential role of GLP-1 in long term weight management in humans (mainly by reducing food intake and improving insulin sensitivity). Another proposed mechanism for the effect of SCFA on inducing satiety is through the stimulation of secretion of PYY in rodents (Psichas et al. 2014). This might be relevant to obese humans since it was demonstrated that levels of PYY are low in obese individuals. Also, PYY levels have been inversely correlated to changes in body weight (Guo et al. 2006). Therefore, PYY might present an important strategy to help decreasing obesity over the long term. PYY has been also shown to be implicated in improving insulin sensitivity through the increase of glucose uptake in muscle (Den Besten et al. 2013), proposing another implication of this hormone in body weight control. It is then important to elucidate the effects of SCFA on PYY and GLP-1 in humans as well as the mechanisms involved.

In addition to the nature and concentration of cocoa flavanols in cocoa/dark chocolate, the degree of polymerization of flavanols (and hence the size) has been suggested to affect the bioactivity of these components, and consequently influence their outcomes on insulin and glucose metabolism. For instance, cinnamtannin A2 (a procyanidin oligomer) has been shown to cause a greater activation of insulin

signalling compared to epicatechin and other procyanidins, and to inhibit GLP-1 in mice (Yamashita et al. 2013). Additionally, a long term study investigating the effects of different types of flavanols (based on the degree of polymerization) in mice fed a high fat diet for 12 weeks, showed that mice administered oligomeric cocoa procyanidins have prevented IR and impaired glucose tolerance to a greater extent than monomeric and polymeric cocoa procyanidins (Dorenkott et al. 2014). A point worth of note is that the bioavailability of procyanidins has been shown to be inversely proportional to procyanidin size, and consequently to the degree of polymerization (Pinent et al. 2012). This could provide an explanation for the inverse association between the degree of polymerization and the inhibition of digestive enzymes (Gu et al. 2011).

In view of these findings, epicatechin, procyanidins as well as quercetin have been deemed to exert beneficial effects on insulin resistance and glucose levels; however the contribution of each of these fractions to the favourable effect remains unclear. Ideally, a study comparing the effects of isolated epicatechin against different polymers of procyanidins and cocoa liquor/dark chocolate might be helpful in identifying the impact of each fraction on the beneficial effect on insulin and glucose metabolism. This will help in making future recommendations based on the fraction/amount of flavonoids in chocolate.

In addition to the polyphenol/flavonoid implication, the insulinogenic effect of cocoa might be attributed to other compounds, as suggested by an experimental study which included 11 healthy men and women. Participants were provided six pairs of foods, each pair containing the same type of food, but one was flavoured with chocolate and the other was not. There was no difference in the glycemic index or in the macronutrient content of foods in each pair. Results showed that chocolate elicited a higher postprandial insulin secretion than other flavourings ($p<0.05$) (Brand-Miller et al. 2003). This higher insulin secretion was explained by the presence of arginine and other mixtures of amino acids in cocoa, which have been known to stimulate the secretion of insulin when consumed with carbohydrates (Van Haeften et al. 1989). Other reasons might also be the palatability of chocolate which may stimulate a pavlovian response, and therefore trigger insulin secretion,

particularly in people who have a preference for chocolate (Brand-Miller et al. 2003).

Studies on diabetic or glucose intolerant animals and humans with cardiovascular risk are still conflicting regarding the association between the consumption of cocoa/dark chocolate and IR and/or glucose levels. Cocoa extracts have been shown to decrease blood glucose levels in diabetic rats (Ruzaidi et al. 2005) and mice (Tomaru et al. 2007) in a dose dependent manner, and to decrease glucose and insulin levels in rats (Jalil et al. 2008). In addition, cocoa supplementation has been effective in counteracting the effects of induced obesity on IR in rats (Gu et al. 2013). In humans with CVD or type 2 diabetes, a crossover study documented that two weeks of DC supplementation rich in polyphenols (1080 mg) to 19 participants with essential hypertension and impaired glucose tolerance, decreases HOMA-IR (Homeostasis modelling assessment-IR) ($p<0.05$), and increases QUICKI (quantitative insulin sensitivity check index) ($p = 0.001$) (both being indicators of insulin sensitivity). However, no changes in these parameters were noticed after the consumption of white chocolate free of flavanols. The improvement of IR was accompanied by a decrease in SBP and DBP (Grassi et al. 2008). Also, Curtis et al. (2012) included 93 diabetic men and women in a parallel study, for whom he administered 27 g of either a placebo DC (with no flavonoids) or a flavonoid-rich DC (containing 850 mg of total flavan-3-ols and 100mg of isoflavones) daily for a period of one year, and noted a decrease in HOMA-IR ($p=0.004$), and an increase in QUICKI ($p=0.04$) in the group administered DC enriched in flavonoids. In addition, Desideri et al. (2012) included 90 elderly individuals for whom he administered doses of cocoa flavanols ranging from 45 mg to 990 mg for 8 weeks, and demonstrated a significant decrease in IR following the intervention ($p < 0.0001$). However, a crossover study undertaken on hypertensives treated with cocoa drink rich in flavanols (containing 900 mg of flavanols) for 2 weeks, did not show an improvement in insulin resistance (assessed by QUICKI) ($p=0.54$) or glucose levels ($p=0.11$). There was only an increase in insulin-stimulated vasodilatation of the brachial artery ($p<0.04$) (Muniyappa et al. 2008). Also, Balzer et al. (2008) showed that in diabetic patients, blood glucose levels were not affected by 30 days of daily

flavanol-rich cocoa supplementation (321 mg, $p = 0.44$), but HbA1C was reduced at the end of the intervention (p= 0.0038). Moreover, a crossover study including 42 volunteers at high risk of cardiovascular diseases (having diabetes and/or hypertension, high cholesterol levels, obesity and family history of premature CHD), who were assigned to consume a skimmed milk drink with or without cocoa powder (containing 495.2 mg of polyphenols) for period of 4 weeks (and separated by 2 weeks of washout period), showed no significant decrease in glucose levels following the consumption of cocoa and skimmed milk (p= 0.18) (Monagas et al. 2009). Lastly, Taubert et al. (2007) failed to show favourable effects on insulin sensitivity or glucose levels in prehypertensive patients following the consumption of 6.3g of DC (containing 30mg of polyphenols) for 18 months (p=0.82) (Taubert et al. 2007). A similar outcome was noticed by the study of Mellor et al. (2010) (after 8 weeks of daily chocolate consumption (45g) containing 16.6 mg of epicatechin) undertaken on diabetics.

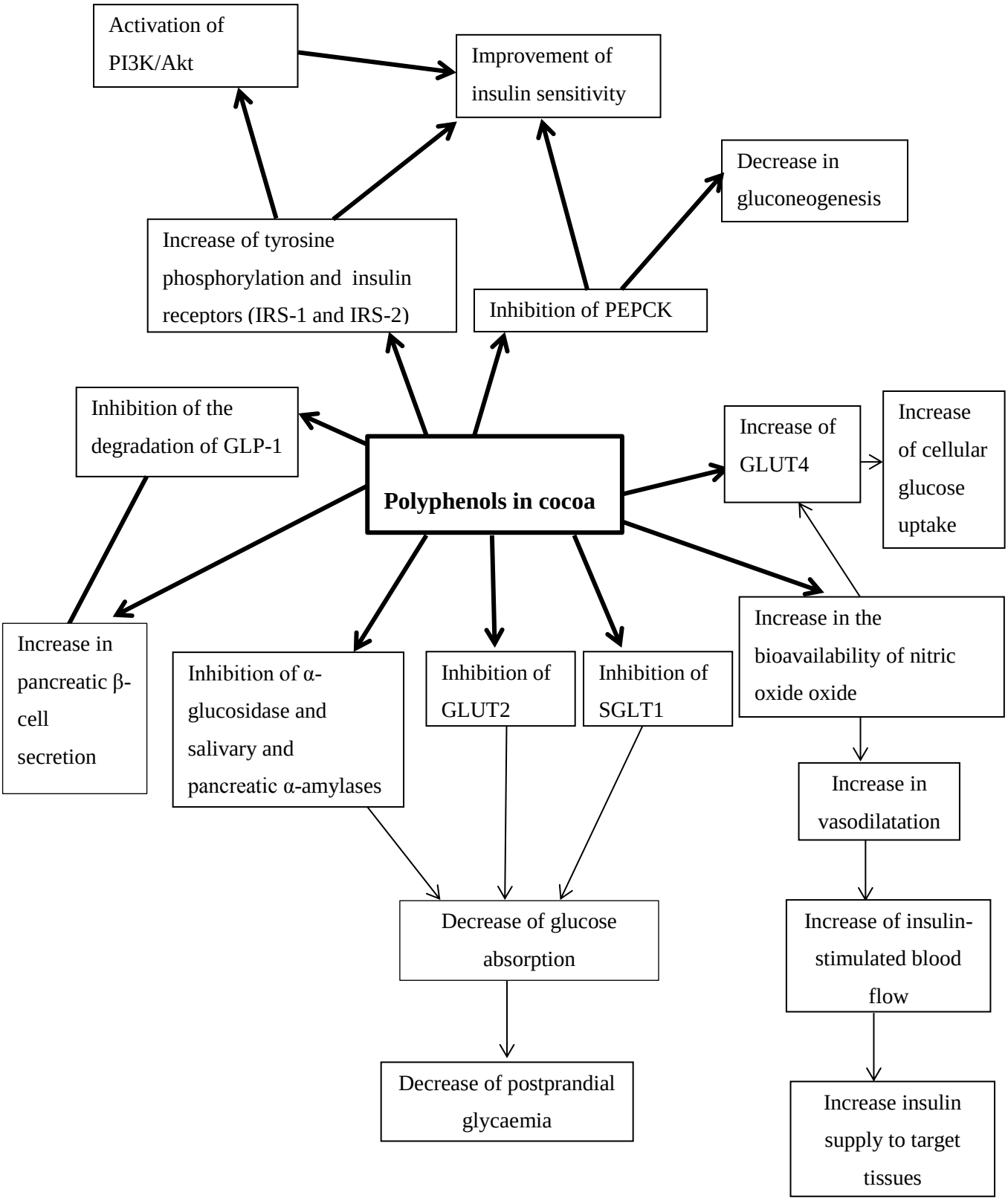



**Figure 1.19: Possible mechanisms by which cocoa polyphenols exert a beneficial effect on insulin and glucose metabolism**

*IRS: Insulin receptor substrate, GLP-1: Glucagon-like peptide 1, PEPCK: Phosphoenolpyruvate carboxykinase, PI3K/AKT: Phosphatidylinositol 3-kinase/protein kinase B, SGLT-1: Sodium-dependent glucose transporter-1, GLUT: glucose transporter. Mechanisms were mainly determined via animal and in vitro studies.*

To our knowledge, only five studies have investigated the effect of daily polyphenol-rich dark chocolate on insulin sensitivity in adults with no history of hypertension, diabetes and CVD. A description of these studies is presented in Table 1.7. A crossover trial included 15 healthy adults who were assigned to receive either dark chocolate or white chocolate for a period of fifteen days each, showed a significant decrease in insulin levels, HOMA-IR as well as an increase in QUICKI only after the consumption of DC (Grassi et al. 2005). In addition, another crossover study including 42 female volunteers recruited according to BMI (BMI<25 Kg/$m^2$ and BMI≥25 Kg/$m^2$), showed that 20g of dark chocolate containing 500 mg of polyphenols decreased HOMA-IR and an increase in QUICKI only in overweight females after 4 weeks. Interestingly, there was an increase in HOMA-IR (p= 0.03), glucose levels (p=0.002), and a decrease in QUICKI (p= 0.033) in overweight females when assigned the placebo dark chocolate (with negligible amount of polyphenols) at the end of the intervention. It has been suggested that overweight individuals might be more affected by the unfavourable effects of DC deficient in polyphenols compared to normal weight individuals (Al Moosawi et al. 2012). Moreover, a study carried out on 49 overweight and obese adults showed a decrease in insulin resistance (assessed by HOMA-IR) after 12 weeks of high flavanol cocoa consumption (Davison et al. 2008). However, a recent study did not show an improvement in HOMA-IR after 7 days of DC supplementation in normal weight women (Di Renzo et al. 2013). Furthermore, no significant effect on insulin levels and HOMA-IR was noticed by a short-term study (5 days) in which twenty overweight and obese participants were assigned to consume cocoa beverages (containing 30 – 900 mg of flavanols). The latter two studies suggest that the short intake of cocoa flavanols may not exert positive effects on insulin and glucose levels (Stote et al. 2012).

Importantly, there was no influence of the type of cacao product used (cocoa or DC) on the results. Both products have been shown to exert beneficial effects on insulin metabolism, while other studies showed no significant effect of cocoa and DC on IR (Table 1.7). Yet, the limited studies suggest the need for further investigations to identify any potential difference in the response based on the use of cocoa or DC.

Regarding glucose levels, the supplementation of DC containing 500 mg of polyphenols significantly decreased glucose levels after 2 weeks (from 4.42 ± 0.7 mmol/l to 3.97 ± 0.54 mmol/l) (Al Moosawi et al. 2010) and 4 weeks (by 0.58 ± 0.68 mmol/l) (Al Moosawi et al. 2012). The latter study noted a decrease in glucose levels only in individuals with BMI>25 Kg/$m^2$, but not in normal weight individuals. In the latter two studies, the decrease in glucose levels was small, which raises questions about the clinical relevance of these outcomes. Nevertheless, a study including 44 overweight individuals (BMI between 25-35 Kg/$m^2$), did not report a significant amelioration in glucose levels following 6 weeks of daily consumption of cocoa drinks containing 805 mg of flavanols (Njike et al. 2011). Stote et al. (2012) also showed no significant effect of cocoa rich in flavanols on glucose metabolism. Although a recent study showed a significant lowering effect of cocoa consumption (30 g daily) on glucose levels ($p = 0.029$), the product contained a higher amount of dietary fibre (33.9 %), which have possibly influenced the results (Sarria et al. 2013).

It is worth mentioning that the results of the meta-analyses of cocoa/DC on glucose and insulin resistance/sensitivity were most likely to show a beneficial effect of these products on IR but not on glucose levels. The meta-analysis of Shrime et al. (2011) included five studies (with healthy participants and patients), and reported a significant decrease in HOMA-IR (-0.94; 95% CI: -0.59, -1.29, $p < 0.001$)), with no significant effect on glucose levels and QUICKI. Similar results were noted in another meta-analysis of 6 trials (including healthy participants and patients), which documented that cocoa/DC supplementation induced a significant decrease in HOMA-IR (-0.67 µIU/ml; 95% CI: -0.98, -0.36), but had no effect on blood glucose levels or QUICKI, suggesting that the favourable effect of cocoa/DC on IR mainly occurs through the decrease in insulin secretion (Hooper et al. 2012). Yet, the non significant effect noted on QUICKI deserves more investigation.

**Table 1.7: Characteristics and outcomes of studies analysing the effect of polyphenol-rich cocoa/chocolate on insulin resistance/insulin sensitivity**

| Study | Design | Intervention | Participants | Duration | Outcomes | Signifi-cance |
|---|---|---|---|---|---|---|
| Grassi et al. 2005 | Randomized crossover, open label study | 100g of **DC** with 500mg of polyphenols v/s 100g of white chocolate with no polyphenols | 7 men & 8 women with BMI:22.6±3.0 kg/m$^2$ 33.9±7.6 years | 15 days of DC and white chocolate separated by a 7-day washout period | ↘ HOMA-IR<br>↗ QUICKI<br>↘ Fasting insulin levels | P<0.001<br>P=0.001<br>P<0.0001 |
| Davison et al. 2008 | Randomized parallel double blinded study | **Cocoa** drink with 902mg of flavanols v/s 36mg of flavanols | 18 men, 31 women BMI:32.8±1.1 kg/m$^2$ 44.4±4.4 years | 12 weeks of cocoa rich in flavanols or low in flavanols | ↘ HOMA-IR<br>↘ Fasting insulin levels | P<0.005 |
| Al Moosawi et al. 2012 | Randomized crossover single blinded study | 20g of **DC** with 500mg of polyphenols v/s 20g of DC with no polyphenols (placebo) | 21 men (BMI<25 kg/m$^2$), 21 women (BMI>25 kg/m$^2$) | 4 weeks of DC and placebo DC separated by a 7-day washout period | ↘ HOMA-IR<br>↗ QUICKI only in BMI>25 kg/m$^2$)<br>No changes in fasting insulin levels | P=0.041<br>P=0.033 |
| Stote et al. 2012 | Randomized crossover single blinded study | 28g of **cocoa** with four different doses of polyphenols (30, 180, 400 and 900mg of flavanols) | 10 men, 10 women 46 ± 2.3 years BMI 36.8±1.0 kg/m$^2$ | 5 days of each dose of flavanols separated by a 10-day washout period | No changes in HOMA-IR, QUICKI or insulin levels | p>0.05 |
| Di Renzo et al. 2013 | Pilot study | 100g of **DC** with 2000mg of polyphenols | 15 women (BMI<25 kg/m$^2$) | 1 week of daily DC supplementation No control group | No changes in HOMA-IR or fasting insulin levels | P>0.05 |

*DC: Dark chocolate, IR: insulin resistance, HOMA-IR: Homeostasis modelling assessment-IR, QUICKI: Quantitative insulin sensitivity check index.*

## 1.6 Effects of cocoa/dark chocolate on lipid profile

Data are still inconclusive regarding the link between cocoa/dark chocolate and serum lipids levels. In individuals with cardiovascular risk, Balzer et al. (2008) showed an inverse association between DC and LDL levels in normolipemic diabetics after ingestion of cocoa rich in flavanols (325 mg) for 4 weeks (by 7.82 %, $p= 0.0063$). Mellor et al. (2010) reported an increase in HDL in diabetics administered PRDC for 8 weeks (by 6.8%, $p= 0.04$), with no significant changes in other serum lipid levels. In the hypertensive population, the consumption of 100g of chocolate rich in polyphenols (500 mg) for 2 weeks decreased total cholesterol by 7 % , and LDL by 12 % ($p<0.05$) (Grassi et al. 2005b). As for hypercholesterolemic individuals, a decrease in serum cholesterol and LDL levels (by 2% and 5.8%, respectively, $p<0.05$) was noted after the consumption of DC bars (containing 360 mg of flavanols) daily for a period of 4 weeks ($p<0.05$) (Allen et al. 2007). In participants with high baseline LDL administered cocoa with doses of polyphenols ranging from 140 mg to 281 mg, serum LDL levels decreased (by 4 - 5 %, $p< 0.05$) and HDL levels increased (by 4.7 - 8%, $p= 0.055$) in a dose dependent manner (Baba et al. 2007a).

As for studies carried out on participants with no cardiovascular risk, results have been controversial. There was no significant change in serum lipids (TC, TG, HDL and LDL) following the consumption of dark chocolate containing 500 mg of polyphenols for a period of 2 weeks (Al Moosawi et al. 2010), or 4 weeks (Al Moosawi et al. 2012). Likewise, several studies have not identified a significant change in lipid profile in healthy subjects after the consumption of cocoa or DC for a period ranging from 2-6 weeks (Mathur et al. 2002; Engler et al. 2004, Grassi et al. 2005; Muniyappa et al. 2008; Nogueira et al. 2012; West et al. 2013; Ibero-Baraibar et al. 2014). Similar results were reported by the study of Neufingerl et al. (2013), which suggested that the HDL increasing effect and the LDL lowering effect of cocoa are mainly attributed to a high amount theobromine (850 mg of theobromine in this study), rather than to the implication of polyphenols.

On the other hand, few studies have shown an improvement in serum lipid levels following cocoa/DC supplementation in individuals with no disease risk. Baba et al. (2007b) administered a drink containing either cocoa powder and sugar (intervention group) (with a total of 766.1 mg of polyphenols) or a sugar drink (placebo group) to 23 healthy subjects for 12 weeks, and showed a significant increase in HDL levels by 24%, and a decrease in LDL levels by 12.6% in subjects assigned the cocoa powder drink ($p<0.001$). Also, a crossover study documented that the consumption of dark chocolate and cocoa powder (containing a total 466mg of flavanols) for 4 weeks results in an increase in HDL by 4 % (by 0.05 mmol/l) ($p = 0.02$) (Wan et al. 2001). Additionally, a study performed on 45 healthy volunteers aged from 19-49 years, showed that the intake of 75g of dark chocolate rich in polyphenols (418 mg of polyphenols) for a 3-week period induced an increase in HDL by 14% (Mursu et al. 2004). The contribution of flavanols to the hypolipemic effect is reinforced by the study of Fraga et al. (2005), which reported that the daily consumption of milk chocolate containing 168 mg of flavanols by 28 healthy male soccer players decreased TC by 11% ($p = 0.009$) and LDL levels by 15 % ($p = 0.02$), while there was no change in HDL levels at the end of the 2-week intervention ($p = 0.2$) (Fraga et al. 2005). It is worth pointing out that the significant lowering effects of cocoa/DC on serum lipids noted in trials were mostly clinically relevant. This is based on previous studies which stated that a decrease in cardiovascular risk is observed when LDL levels drop by 1% (Jia et al. 2010), and HDL levels increase by 10 mg/dl (Ali et al. 2012) (0.026 mmol/l).

The previous results, suggest that, even with the considerable lower amount of fat in cocoa compared to DC, both products have been reported to lower serum lipid levels. A meta-analysis stratifying the studies according to the type of cacao product may be more reliable in showing differences in response to intervention based on the administration of cocoa or DC.

Several meta-analyses aiming to review the effect of cocoa/DC on serum lipids have been performed, and included studies on normo and hypercholesterolemic individuals. The meta-analysis of Jia et al. (2010) included 8 studies and noted a significant decrease in LDL levels (-5.87 mg/dl (95% CI; -11.13, -0.61, $p<0.05$))

following cocoa/DC consumption, with a marginal decrease in TC levels that did not reach statistical significance (-5.82 mg/dl; 95% CI: -12.39, 0.76, $p= 0.08$). A meta-analysis of 10 studies reported similar results: serum TC (- 6.23 mg/dl (95% CI: -11.6, -0.85, $p<0.05$) and LDL levels (- 5.9 mg/dl; 95% CI: -10.47, -1.32, $p< 0.05$) decreased, whereas no significant effects on HDL and TG levels were noted (Tokede et al. 2010). However, in both meta-analysis, heterogeneity was described as a drawback, and the beneficial effect on lipid profile was no longer significant when low quality studies were excluded (Jia et al. 2010; Tokede et al. 2011). Moreover, it seems that studies with short duration (2 weeks) were more effective in decreasing lipid levels than longer term studies. This was mainly explained by the decrease in adherence observed in long-term studies (Tokede et al. 2011). The baseline health status is also predictive of the outcomes. A decrease in LDL and TC levels was only noted in patients with high cardiovascular risk but not in healthy subjects (Jia et al. 2010; Tokede et al. 2010). This effect is plausible due to the fact that individuals with cardiovascular risk have usually dyslipidemia or abnormalities in lipid metabolism (Nathan 1999); hence it is more likely that their serum lipid levels will be improved. Moreover, flavanol doses less than 500 mg seem to be more effective in lowering LDL levels than doses higher than 500 mg (Tokede et al. 2011). However, Jia et al. (2010) showed a significant decrease in TC and LDL levels only when doses of flavanols were less than 260 mg. This may be due to the fact that a high amount of polyphenols can counteract the benefits of polyphenols on lipid profile (Jia et al. 2010). This outcome corresponds with a finding of an animal study which showed that cocoa may lessen plaque formation at low doses, while it did not cause this effect at high cocoa doses (Vinson et al. 2006). Yet, the optimal dose of polyphenols supposed to have beneficial effects on lipid profile has not been yet determined (Jia et al. 2010).

The mechanisms by which cocoa/DC exert beneficial effects on lipid profile have not been clearly elucidated. Flavonoids were shown to inhibit the intestinal absorption of cholesterol in rats (Ikeda et al. 1992), to increase the hepatic expression of LDL receptors (Pal et al. 2003), and to inhibit the synthesis of LDL (Wilcox et al. 2001) in human hepatic cells. An in vitro study showed that

polyphenols activate AMPKα1 and AMPKα2 phosphorylation in human hepatic cells, which resulted in a decrease in lipid accumulation in these cells (Zang et al. 2006). It was also shown that in rats fed a high cholesterol diet, cocoa powder was able to increase the faecal excretion of cholesterol (Baba et al. 2007a). The polyphenol metabolites in the colon might also be involved in decreasing cholesterol levels. For instance, it was shown that 3-dihydroxytoluene, a flavanol metabolite produced in the colon, inhibits the incorporation of acetate into cholesterol in the liver cells of rats, and therefore inhibits cholesterol synthesis (Glässer et al. 2002). Yet, these mechanisms have not been supported in human studies. A decrease in ApoB (the major LDL and VLDL protein) and a subsequent decline in serum LDL levels were albeit noted in humans (Baba et al. 2007a).

Finally, it was hypothesized that there might be other components in DC responsible for the potential beneficial effects. For instance, in addition to the effect of theobromine, cocoa butter is an important source of oleic acid, which exerts a positive effect on lipid profile (Corti et al. 2009). Moreover, a study reported that substituting a carbohydrate snack with a milk chocolate bar resulted in an increase in serum HDL levels and a decrease in TG levels. This study suggested that refraining from eating the carbohydrate snack exerted beneficial effects on lipid levels. These effects were not particularly related to chocolate consumption (Kris-Etherton et al. 2002).

## 1.7 Effects of cocoa/dark chocolate on blood pressure

The link between cocoa and blood pressure has been first identified from research undertaken on the Kuna Indians of Panama, who were known to consume large quantities of cocoa (at least three 10-oz servings of cocoa per day) (McCullough et al. 2006). This amount provides more than 900 mg of flavanols daily (Bayard et al. 2007). The kuna Indians had low incidence of high blood pressure despite their high sodium intake (McCullough et al. 2006), with a mean SBP/DBP around 110/70 mmHg (Hollenberg 2006). Researchers suggested this protective effect is not genetic, as migration of the Kuna Indians to the urban areas has increased the prevalence of hypertension among them. This was explained by the change in their

dietary habits and lifestyle, such as the increase in smoking status and stress levels, and particularly the decrease in their cocoa intake, which became 10-fold lower than the Indians living in the indigenous islands (McCullough et al. 2006).

The beneficial effect of cocoa on blood pressure was primarily reported in a correlational study on 470 elderly men with no history of CVD. Men with the highest tertile of cocoa intake (4.18 g/day) demonstrated a lower SBP ($p=0.0008$) and DBP ($p=0.0001$) than men in the lowest tertile of intake (no cocoa consumption) (Buijsse et al. 2006).

Many intervention studies were carried out on cocoa/DC and blood pressure, mainly in the hypertensive population. DC supplementation was mostly effective in decreasing blood pressure in hypertensives administered doses of flavanols ranging from 300-1052 mg for a period of 2-6 weeks (Taubert et al. 2003; Grassi et al. 2008; Davison et al. 2010; Heiss et al. 2010; D'El-Rei et al. 2013). Other studies, on the other hand, did not report a significant effect of cocoa/DC on blood pressure (Muniyappa et al. 2008; Ried et al. 2009).

Nonetheless, clinical trials studying the effect of dark chocolate or cocoa on blood pressure in adults with no type 2 diabetes, hypertension and CVD are still conflicting. Al Faris (2008) reported a significant decrease in SBP (from 115.9 ±12.6 mm Hg to 107.5 ± 8.6 mm Hg), and DBP (from 73 ± 9.9 mmHg to 67.7 ±9.7 mmHg) following the consumption of 100 g of dark chocolate rich in polyphenols (500 mg) for two weeks ($p<0.05$) (Al-Faris 2008). Similar results were documented by Al Moosawi et al. (2010): a decrease in SBP (from 119.38 ± 10.51 mm Hg at baseline to 112.4 ± 9.51 mm Hg) and DBP (from 78.62 ± 7.74 mm Hg at baseline to 73 ± 5.06 mm Hg) after 2 weeks of daily DC consumption (20g) containing 500 mg of polyphenols ($p<0.05$) (Al Moosawi et al. 2010). However, in a crossover study, Grassi et al. (2005) demonstrated a decrease in SBP after consuming 100 g of DC (500 mg of polyphenols) for 2 weeks (107.5 ± 8.6 mmHg after DC consumption versus 113.9 ± 8.4 mm Hg after white chocolate group at the end of the study, $p<0.05$), while DBP did not significantly change. In line with these findings, 500 mg of polyphenols in DC were effective in decreasing SBP in normal weight women by 3.41 ± 6.08 mmHg and by 4.48 ± 7.82 mmHg in overweight women ($p<0.05$), while

the effect on DBP was only positive in overweight women (DBP significantly decreased by 3.22 ± 3.75 mmHg). It was documented that overweight females better respond to the DC blood pressure lowering properties (Al Moosawi et al. 2012). Fraga et al. (2005) reported that 168 mg of flavanols induced a significant decrease in DBP by 5 mm Hg (p= 0.01), while the decrease in SBP did not reach statistical significance (p= 0.06). Similar results were reported by a study undertaken on 49 overweight and obese participants who were randomly assigned to receive a high flavanol cocoa drink (containing 902 mg of flavanols) or low flavanol cocoa drink (36 mg of polyphenols) for a period of 12 weeks. In the high flavanol cocoa drink, a decrease in DBP by 1.6 mm Hg (p= 0.04) with no change in SBP was noted (Davison et al. 2008). As it has been shown that clinically relevant reductions in blood pressure can be considered when changes exceed 5 mmHg (Glynn et al. 2002), most of the significant changes in blood pressure noted in previous studies were also clinically relevant.

However, several studies failed to report a lowering effect of cocoa/DC on BP (blood pressure) in normotensive individuals. The trial of Murphy et al. (2003) did not note a significant effect on BP in participants administered 234 mg of flavanols and procyanidins. Same outcome was noticed in the studies of Engler et al. (2004) (259 mg of flavonoids), Crews et al. (2008) (754.71 mg of proanthocyanidins), Shiina et al. (2007) (550 mg of flavanols), Njike et al. (2011) (805 mg of flavanols), Neufingerl et al. (2013) (325 mg of flavonoids) and (Di Renzo et al. 2013) (2000 mg of polyphenols).

In view of these conflicting outcomes, the results of meta-analyses might be effective in analysing inconsistencies and generalizing results. Few meta-analyses looked at the effect of cocoa/DC on blood pressure (A summary is provided in table 1.8).

**Table 1.8: Characteristics of meta-analyses assessing the effect of cocoa/DC on blood pressure**

| Meta-analysis | Number of studies | Baseline health Status in studies | Active ingredients polyphenols range (mg) | Duration | Results |
|---|---|---|---|---|---|
| Taubert et al. 2007 | 5 | 3 normotensives/ 2 hypertensives | 213 – 500 | 2 weeks | SBP (-4.7 mmHg) (p=0.002) DBP (-2.8 mmHg) (p=0.006) |
| Desch et al. 2010 | 10 | 5 normotensives/ 5 hypertensives | 30-902 | 2-18 weeks | SBP (-4.75mmHg) DBP (-2.5 mmHg) (p<0.001) |
| Ried et al. 2010 | 13 | 10 normotensive/ 3 hypertensives | 30-1008 | 2-18 weeks | SBP (-3.2mmHg) p=0.001) DBP (-2.0 mmHg) (p=0.003) |
| Shrime et al. 2011 | 22 | 11 normotensive/ 11 hypertensives | 30-1080 | 2-18 weeks | SBP (-1.63 mmHg) p=0.033) No change in DBP |
| Hooper et al. 2012 | 22 | 10 normotensive/ 12 hypertensives | 30-1080 | 2-18 weeks | No change in SBP DBP (-1.6 mmHg (p<0.05) |
| Ried et al. 2012 | 20 | 10 normotensive/ 10 hypertensives | 30-1080mg | 2-18 weeks | SBP (-2.77 mmHg) p=0.005) DBP (-2.2 mmHg) (p=0.0006) |

*SBP: Systolic blood pressure, DBP: Diastolic blood pressure*

As showed in table 1.8, the majority of meta-analyses showed a potential effect of cocoa/DC on decreasing blood pressure. However, the results are limited by the large heterogeneity of the studies. This includes a difference in the number of subjects, amount of polyphenols (flavonoids) and the study duration. In addition, most of the studies lasted for 2-8 weeks, while Taubert et al. (2007) is the only study that lasted for 18 weeks. Yet, excluding the latter study did not significantly affect the results (Ried et al. 2012).

While two meta-analysis showed no correlation between polyphenol dose, duration, BMI and the change in blood pressure (Ried et al. (2010), another meta-analysis concluded that studies lasting 2 weeks showed a significant decrease in blood pressure, while studies lasting for a longer period did not note this effect. In addition, the stratification of studies according to the amount of sugar present in the cocoa/dark chocolate product had an influence on the results. This was demonstrated in the meta-analysis of Ried et al. (2012), which shows that low sugar content in the experimental cocoa/DC (less than 10g/day) had a larger beneficial effect than the ones using higher amounts of sugar. This meta-analysis also showed a more pronounced effect on blood pressure when participants were younger, and when a polyphenol-free placebo product was used instead of a placebo product low in polyphenols (Ried et al. 2012).
Nevertheless, the meta-analysis of Ried et al. (2010; 2012) showed that that cocoa/DC was effective in decreasing blood pressure in pre-hypertensive and hypertensive subjects, but not in normotensive subjects. No other meta-analyses stratified participants according to their health status, and tested the influence of this factor on blood pressure results after cocoa/DC supplementation.

In the presence of a beneficial effect, the dose and fraction of flavanols supposed to lower blood pressure is still unclear. It was recently suggested by the EFSA that the consumption of 200 mg of flavanols daily might have a beneficial effect on endothelium-dependent vasodilation, and could possibly result in the decrease in blood pressure. This effect could be achieved by the consumption of 10g of flavanol-rich chocolate or 2.5g of high-flavanol cocoa (EFSA 2012). As for the flavanol fraction, a meta-analysis of 70 studies showed that epicatechin is the main flavonoid responsible for decreasing blood pressure. It was reported that the consumption of chocolate or cocoa containing 25 mg of epicatechin was effective in deceasing SBP by 4.1 mm Hg and DBP by 2 mm Hg (Ellinger et al. 2012). However, the meta-analysis of Hooper et al. (2012) documented a significant effect on SBP and DBP only when the dose of epicatechin was higher than 50 mg. In particular,
–(-) epicatechin is the main stereoisomer responsible for an effect on NO and vasodilatation, unlike +(-) epicatechin and –(-)catechin (Ottaviani et al. 2011).

Procyanidins were also shown to exhibit vasodilative and blood pressure lowering effects (Taubert et al. 2007). Indeed, the fact that a meta-analysis showed a lowering effect of cocoa but not tea on blood pressure was suggested to be due to the difference in polyphenol composition between cocoa and tea; the blood pressure lowering effect might be attributed to the procyanidin fraction of flavanols that is abundant in cocoa, but not in tea (Taubert et al. 2007).

The mechanism underlying the effect of dark chocolate/cocoa on blood pressure may be explained by the increased availability of nitric oxide and the subsequent increase in vasodilatation (Grassi et al. 2005). However, a vasodilative effect does not necessarily imply a blood pressure lowering effect unless it simultaneously decreases the renal pressure threshold responsible for sodium homeostasis (Egan et al. 2010). This might actually explain the mechanism by which cocoa polyphenols improve insulin sensitivity and endothelial function through vasodilatation, but do not exert blood pressure lowering effects (Grassi et al. 2005; Wang-Polagruto et al. 2006; Balzer et al. 2008). Cocoa flavanols can also raise vasodilatation independently of NO, by increasing substances like prostacyclin and endothelium-derived hyperpolarizing factor (Corti et al. 2009). Another proposed mechanism is the implication of the Renin-Angiotensin system through the inhibition of the ACE, suggesting that foods rich in flavonoids can mimic the effect of ACE inhibitors without the medications causing side effects (Actis-Goretta et al. 2003; Lavoie and Sigmund 2003). This happens by a competition between flavonoids and the substrate for the active site of the enzyme (Actis-Goretta et al. 2003). However, this theory was doubted due to the low concentration of epicatechin achieved in the human plasma (nanomolar range) (Baba et al. 2000; Holt et al. 2002), which makes it less likely to inhibit ACE. Furthermore, the associated decrease of S-nitroglutathione (a vasodilatative) with BP after DC consumption, suggests an implication of this marker in lowering blood pressure (Taubert et al. 2007).

Interestingly, the lowering effect on blood pressure might also be caused by a "placebo effect" as explained by the meta-analysis by Egan et al. (2010), which reported a decrease in blood pressure in 6 of 7 open-label studies, but only in 1 of 6 double-blind studies. This suggests that the positive outcome might be explained by

individual expectations of a positive effect. The most plausible evidence is the study undertaken by Taubert et al. (2007), which reported that a dose of 30 mg of polyphenols is effective in decreasing BP. This dose, considered negligible, might have led to a decrease in BP due to the use of white chocolate in the placebo group, thus participants were aware of the fact that they constituted the intervention group.

Lastly, the non significant effect of cocoa polyphenols on blood pressure has also few explanations: It was suggested that –(-)catechin (which is produced by epimerization of epicatechin during processing of cacao beans) may counteract the blood pressure lowering effects of –(-)epicatechin (Egan et al. 2010). Also, the dose of flavanols required to induce changes in blood pressure might be high. For instance, Davison et al. (2010) showed that a dose of flavanols equivalent to 1052 mg can reduce blood pressure in participants with mild hypertension, while the doses of 33, 372 and 712 mg did not induce such effect. This matches with the fact that cocoa powder consumed by the Kuna indians contained 3.6% of flavanols, while the DC in the market usually contains 0.5 % of flavanols (Chevaux et al. 2001), which might be less likely due induce a decrease in blood pressure.

### 1.8 Effects of cocoa/dark chocolate on glucocorticoid metabolism

Limited research previously tested the effects of polyphenols on glucocorticoid metabolism, and very few studies assessed the effects of cocoa polyphenols on cortisol and cortisone levels. Glucocorticoid metabolism plays a particular role in the MS (Masuzaki et al. 2001), thus identifying beneficial effects on cortisol levels might help in preventing or delaying the occurrence of MS. Green tea polyphenols were shown to inhibit glucocorticoid gene expression (Abe et al. 2001). Similar effects were noticed by another study on chlorogenic acid (Arion et al. 1997). Also, tea polyphenols exerted an inhibitory effect on 11 β HSD 1 (Hindzpeter et al. 2014), and flavonone derivatives were potent inhibitors of this enzyme (Miguet et al. 2006). These results suggest a potential effect of polyphenols on cortisol metabolism, yet it is important to be aware of the different composition of the polyphenol fraction in cocoa compared to the other polyphenols tested.

Regarding research on DC, a study including 30 subjects assigned to receive 40g of DC for 2 weeks, showed a decrease in the urinary excretion of cortisol along with a decrease in urinary stress hormones (adrenaline, noradrenaline and normetanephrine) (Martin et al. 2009). However, a pilot study conducted on overweight and obese subjects did not note a significant change in urinary cortisol and cortisone levels, nor in the cortisone-to-cortisol ratio (indicator of 11 β-HSD1 activity) after 14 days of daily consumption of polyphenol-rich dark chocolate (with 500 mg and 1000 mg doses of polyphenols) (Al Moosawi et al. 2010). Similar results were noted after 4 weeks of PRDC (500g) consumption. However, dark chocolate low in polyphenols increased salivary cortisol in overweight and normal weight females at the end of study (Al Moosawi et al. 2012).

The mechanism responsible for the potential beneficial effect of polyphenols on cortisol metabolism remains unclear. It could be attributed to the fact that polyphenols inhibit Glucose-6-phosphatase activity, and therefore reduce the formation of NADPH, which is responsible for the regeneration of 11β-HSD1. This results in a decrease in the activity of 11β-HSD1, and a subsequent decrease in cortisol levels (Arion et al. 1997). Furthermore, polyphenols were shown to decrease corticosteroid-induced oxidative stress in chicken treated with corticosterone, providing another mechanism by which polyphenols may inhibit oxidative stress and cortisol (Eid et al. 2003). Further studies are needed to elucidate the effect of cocoa polyphenols on glucocorticoid metabolism, as well as the potential mechanisms involved.

## 1.9 Effects of cocoa/dark chocolate on fat metabolism, body weight and obesity

While many human studies reported benefits of DC on cardiovascular health, these effects might be limited by the high fat and sugar content of DC (Miller et al. 2006; McShea et al. 2008). In fact, chocolate provides a considerable high amount of energy, which may lead to weight gain if not introduced in the context of a balanced controlled-calorie diet, suggesting the importance of moderation in avoiding side effects.

However, there has recently been an emerging research on the effects of polyphenols in counteracting obesity, and on the role of polyphenols in body weight control. Dietary polyphenols have been suggested as one of the complementary approaches to manage obesity, due to the ability of some of these components like catechin, curcumin, anthocyanins and resveratrol to modify molecular and physiological pathways implicated in adiposity and energy metabolism (Meydani and Hasan 2010). The implication of polyphenols in body weight control might be particularly important since despite weight-loss interventions, it is estimated that 68 % of the American adult population are still overweight or obese (Flegal et al. 2010). In addition, the modest effect of hypocaloric diets on reducing weight mainly due to poor compliance (Foster et al. 2005), raises the need for strategies that target long-term interventions and may therefore help in long-term weight maintenance.
Green tea polyphenols (mainly catechin) have been previously shown to decrease weight in adults after 6 weeks of daily supplementation (Yang et al. 2012). This effect was not attributed to caffeine, as the supplementation of cocoa tea (a decaffeinated tea plant) to mice has attenuated the adverse effects of a high-fat diet induced obesity, including the increase in body weight and plasma lipid levels (Yang et al. 2013). In addition, lemon flavonoids inhibited body weight gain and fat accumulation in the adipose tissue of mice fed a high fat diet for 12 weeks (Fukuchi et al. 2008). Quercetin-rich onion peel extract was also shown to inhibit adipogenesis in preadipocytes (Moon et al. 2013).
The potential effects of cocoa polyphenols on obesity were mainly studied in animal and in vitro studies, and the contribution of human studies remains relatively limited. A summary of these potential effects is presented in Figure 1.20.

*Animal studies*

While testing the effects of cocoa on several conditions such as atherosclerosis in rats, it was observed a lower adipose tissue weight in rats fed a cocoa-rich diet, compared to those fed a diet free of cocoa, suggesting that cocoa might be implicated in obesity. This led to more focused studies on the effect of cocoa on body weight control. Twenty rats were fed an isocaloric high fat diet including either a real cocoa or a mimetic one (with a similar composition to cocoa in terms of moisture,

carbohydrate, protein, fat and fibre) for a period of 3 weeks. Results showed a lower body weight gain ( by 13.6 %) and mesenteric white adipose tissue (MES-WAT) weight gain in rats fed with a real cocoa compared to rats fed a mimetic one ($p<0.05$) (Matsui et al. 2005). In support of this data, another study undertaken on 44 mice who were divided into 4 groups and administered either a normal diet, a high fat diet, or a high fat diet with 2 different doses of cocoa polyphenol extract (CPE) for a period of 5 weeks, showed less gain in body weight (by 5.8% and 12.6% in a dose dependent manner) and in the liver and epididymal fat mass in mice administered CPE ($p<0.05$) (Min et al. 2012). A recent study also found similar results: a lower body weight gain (by 16%) and WAT weight gain was noted in mice fed a high fat diet with cocoa for 10 weeks, compared to rats fed only with a high fat diet ($p<0.01$) (Gu et al. 2013). Similarly, cocoa liquor procyanidins (CLPr) were shown to reduce body weight gain and fat accumulation in the adipose tissue of obese diabetic mice when provided with a high fat diet for a period 13 weeks ($p<0.05$) (Yamashita et al. 2012), whereas CLPr along with a high fat diet did not attenuate weight gain in diabetic obese mice assigned to a similar protocol for 3 weeks (Tomaru et al. 2007). With the aim of investigating the molecular mechanisms triggering the influence of cocoa on the metabolism of lipids, an analysis of the gene expression in the liver and MES-WAT of rats showed that in the liver, cocoa decreases the expression of genes involved in the synthesis of fatty acids (such as fatty acid synthase), as well as the expression of genes involved in the biosynthesis of cholesterol (such as 7-dehydrocholesterol reductase, squalene synthase and squalene epoxidase) (Matsui et al. 2005). The mechanisms involve the transcription factors PPARs. In MES-WAT, cocoa induces a decrease in the expression of genes implicated in fatty acid transport (like fatty acid transporter and Apo E), along with PPAR-γ, as well as in the expression of genes involved in fatty acid synthesis (such as fatty acid synthase and enoyl-coA hydratase) (Matsui et al. 2005). It was also stated that polyphenols might activate PPAR-γ in hepatocytes leading to an increase in fatty acid oxidation (Goldwasser et al. 2010).

Yamashita et al. (2012) indicated that CLPr may increase the gene and protein expressions of the uncoupling proteins (UCPs). UCP-1 (which plays a role in thermogenesis in the brown adipose tissue), UCP-2 in white adipose tissue, and

UCP-3 in skeletal muscle (which are both involved in the regulation of energy metabolism). The effect on UCPs is explained by the increased phosphorylation of AMPKα, which is involved in the increase in fatty acid oxidation (Yamashita et al. 2012). Gu et al. (2013) also suggested that cocoa powder decreases lipid absorption and leads to a decrease in body weight gain.

Furthermore, the lowering effect on body weight might be suggested to be due to the increase in energy expenditure through the increase in the consumption of oxygen ($VO_2$) and the excretion of carbon dioxide ($VCO_2$). This was noted in an animal study in which rats were supplemented with flavanols extracted from cacao (0.2%) for 2 weeks. This effect was explained by the stimulation of AMPK in skeletal muscle, which is activated by PGC1-α , the latter being involved in thermogenesis (Osakabe 2013).

Moreover, the potential effect of polyphenols on body weight control was suggested to be attributed to the central nervous system through the modulation of neuropeptides implicated in satiety and food intake (Panickar 2012). Research is still very limited regarding this association, but the theory was mainly considered due to the ability of some polyphenols (like epicatechin and resveratrol) to cross the blood brain barrier (Panickar 2012). In fact, mice treated with resveratrol demonstrated a decrease in NPY and AgRP (Agouti-related protein) (a neuropeptide that induces obesity) in a dose dependent manner (Hamao et al. 2011). In addition, decaffeinated coffee rich in chlorogenic acid was shown to increase the postprandial secretion of GLP-1 (Johsnton et al. 2003), which may induce satiety by inhibiting gastric emptying (Flint et al. 1998). Moreover, isoflavone supplementation increased peptide Y (PYY) (satiety hormone that decreases food intake) (Weickert et al. 2013). Studies investigating the effect of cocoa polyphenols on neuroregulatory peptides are needed to identify any potential effect of these polyphenols on satiety.

In view of all the facts exposed above, animal studies provide an important rationale for carrying out human studies analysing the associations between cocoa polyphenols and body weight. However, it is important to acknowledge the lack of evidence of the presence of biologically active BAT in humans, which has explained a part of the weight lowering effect of cocoa polyphenols in rodents through UCP1.

Furthermore, -(-)epicatechin glucuronide, an important metabolite of -(-)epicatechin, has been noted to be differently metabolized between humans and animals (Natsume et al. 2003). This metabolite has been deemed to have a lower antioxidant capacity in humans compared to rats (Natsume et al. 2004). In addition, body weight control differs between free living humans and animals (Liebel 2008), which makes it difficult to compare the results between the 2 species.

*In vitro studies*

Cocoa polyphenol extract (CPE) (obtained from the extraction of polyphenols from 50g of cocoa powder) was reported to inhibit the primary stage of adipogenesis in preadipocytes 3T3 L1 derived from mice, and to decrease the accumulation of lipids in these cells in a dose dependent manner ($p<0.05$). It was shown that CPE inhibits diet-induced adipogenesis in cells by suppressing mitotic clonal expansion, a step that enables DNA remodelling for gene expression during the development of fat cells. The mechanism involves a decrease in mRNA expression of PPAR-γ and C/EBPα (Ccaat-enhancer-binding proteins) (Min et al. 2012) (both being transcription factors in adipogenesis (Rosen et al. 2002), together with the decrease in mRNA expression of fatty acid synthase in these cells (Min et al. 2012). The results might suggest a possible role of cocoa in preventing weight gain in human adults, as studies showed that the expansion of adipose tissue in adults can result not only from increased adipocyte size but also from increased adipocyte number (Gregoire et al. 1998; Gregoire, 2011).
Min et al. (2012) proposed that the inhibitory effect on lipid accumulation may possibly be due to the fact that CPE inhibits insulin receptor kinase activity in adipose tissue, which subsequently suppresses ERK (extracellular signal-regulated kinase). The latter cascade plays an essential role in the differentiation of adipocytes.

Moreover, findings from "in vitro" studies showed that polyphenols from teas and berries inhibit digestive enzymes, providing a justification for investigating the implication of cocoa polyphenols in the digestion of fats and carbohydrates. In fact, a cell study investigated the effect of various doses of cocoa extracts (containing different amounts of polyphenols) on the digestive enzymes: pancreatic α-amylase

(involved in the breakdown of starch), pancreatic lipase (hydrolyzes TG), and pancreatic phospholipase A2 (implicated in the digestion of phospholipids), which were isolated from a porcine pancreas. Results showed that cocoa extracts inhibit the activity of these enzymes in a dose-dependent manner. These studies suggest that cocoa polyphenols may interfere with the breakdown and absorption of carbohydrates and lipids, thus providing one of the mechanisms by which cocoa and DC may reduce obesity. These findings suggest that cocoa/DC might act as anti-obesity agents such as Orlistat by targeting digestive enzymes, and decreasing the absorption of macronutrients (carbohydrates and lipids) (Gu et al. 2011). Yet, the optimal doses supposed to induce this effect in humans remain undetermined.

*Human studies*

Human studies are still unclear on the relationship between cocoa/DC and obesity. Regarding the effects of DC on satiety, a study demonstrated that the ingestion of 30g of dark chocolate (containing 85% of cocoa) by 12 normal weight healthy females increased satiety and decreased hunger, which was verified by scoring appetite on a VAS (visual analogue scale) at different intervals, and up to one hour after consuming the chocolate ($P<0.01$). However, there was no control group in this study, and no significant change in the blood satiety hormone levels CCK (cholecystokinin), ghrelin and GLP-1 compared to baseline was noted. In the second part of this study, females were divided into 2 groups and randomly assigned to either smell the same type of chocolate (experimental group), or perform no smelling (control group). The results showed a decrease in appetite and an inverse correlation between ghrelin and appetite in the experimental group ($p<0.01$) (Massolt et al. 2010). Furthermore, a crossover study including 16 healthy males showed that eating 100g of dark chocolate 2 hours before serving an "ad libithum" meal, decreased food and energy intake by 8% ($p= 0.01$), and promoted higher satiety scores ($p= 0.02$) when compared to eating the same amount of milk chocolate before the "ad libithum" meal on another day. However, since the amount of polyphenols in DC was not specified in the latter two studies, the implication of PPs in inducing satiety is unclear. The satiety effect might also be due to the high cocoa content in dark chocolate, which has an intense flavour that lessens the eating desire (Sorenson and

Astrup 2011). Additionally, the high fat content of dark chocolate (Oliviero et al. 2009) increases gastrointestinal transit time, and may raise the levels of satiety hormones (Samra 2010). However, Massolt et al. (2010) did not note a significant change in satiety hormones levels (like CCK and GLP-1) after DC consumption. Further studies are required to determine the effect of DC on satiety.

Also, a cross-sectional study investigating the effects of chocolate consumption on BMI and including 1018 healthy men and women aged between 20-85 years, showed that with or without adjustment of confounding factors like age, sex, depression, saturated fatty acid, fruit, vegetable, and calorie intakes, individuals with high frequency of chocolate consumption had a lower BMI ($p=0.01$ unadjusted), but surprisingly a higher calorie and saturated fatty acid intake. Moreover, the amount of chocolate consumed was not positively or negatively correlated with BMI (Golomb 2012). Nonetheless, there was no differentiation between the consumption of DC and other types of chocolate, and since this study is cross-sectional, the association between chocolate consumption and BMI cannot be conclusive. In addition, the lower body weight observed in regular chocolate eaters (Golomb 2012) might not be due to the chocolate in particular, but to the sweet snack that reduces cravings and enhances diet satisfaction over the long term. This was demonstrated by a pilot study performed on 26 overweight and obese premenopausal women who were administered either a non-chocolate snack or a dark chocolate snack daily for 18 weeks along with a reduced-calorie diet. The study showed that the two snacks were equally effective in helping to decrease body weight, body fat, and waist and hip circumferences in overweight and obese women ($p <0.001$), as they lower the probability of rebound effect upon re-introducing the food, after restricting it for a certain amount of time (Piehowski et al. 2011). This notion was reinforced by research on eating behaviour, which demonstrated a marginally significant association between exposure to food temptations and a healthier food choice ($p= 0.056$) compared to a control state (Kroese et al. 2009), and can increase body weight control. Another possible explanation to the inverse correlation between chocolate consumption and body weight might be attributed to socio –economic factors, as it has been shown in a cross-sectional study that participants with a high socio-economic level have usually a lower body weight and a more frequent

chocolate consumption compared to people with low socio-economic status (p=0.01) (Hodgson et al. 2008). Hence, it would be important to verify this association in future cross-sectional studies.

In line with the effect of polyphenols on energy expenditure in mice, polyphenol supplementation (resveratrol + EGCG) for 3 days increased energy expenditure (assessed by indirect calorimetry) in 18 overweight volunteers (Most et al. 2013). In addition, epicatechin-rich cocoa increased mitochondrial biogenesis and oxidative phosphorylation in type 2 diabetic patients when administered epicatechin-rich cocoa (100g /day for 3 months) (Taub et al. 2012). Furthermore, the supplementation of a single dose of epicatechin-derived cocoa (1 mg/Kg) showed an increase in fat oxidation in 20 adults, which was demonstrated by a decrease in their respiratory quotient (Gutiérrez-Salmeán et al. 2014). These studies constitute an important rationale for carrying out more studies testing the effect of cocoa polyphenols on energy expenditure in humans.

Additionally, it was documented that DC increases the availability of NO (Field et al. 2011), which enhances adipose tissue lipolysis, inhibits the synthesis of fatty acids, and enhances glucose and fatty acid oxidation (Jobgen et al. 2006). This provides another mechanism for the effect of DC on obesity, and supporting a link between blood pressure, glucose and body weight through a NO-related mechanism. The association between obesity and endothelial function was supported by an animal study, which showed that the supplementation of diabetic Zucker rats with L-arginine for 10 weeks increased the production of NO, and decreased weight gain in this group, while no changes were observed in the group administered alanine (Fu et al. 2005).

In contrast, randomized clinical trials lasting from 2-8 weeks did not report significant changes in weight following the regular consumption of DC with daily amounts ranging from 20-50g a day, and comprising 259-2135mg of polyphenols (West et al. 2013; Al Moosawi et al. 2012; Nogueira et al. 2012; Njike et al. 2011; Al Moosawi et al. 2010; Ried et al. 2009; Allen et al. 2008; Grassi et al. 2005;

Engler et al. 2004). However, a recent study showed that 7 days of cocoa supplementation with 2000 mg of polyphenols did not significantly affect BMI but resulted in a decrease in waist circumference ($p \leq 0.05$). (Di Renzo et al. 2013). This is probably the only study that documented such results, which significance remains under question due to the short study duration and the absence of a control group. The fact that BMI did not significantly change in the previous studies might be due to the study duration, which was not sufficient to identify changes in anthropometric measures. Also, all the previous studies had in common the assessment of anthropometric measures as a part of a routine measurement and not as a primary outcome in studies, hence it might not have been an emphasis on replacing cocoa/DC by another food of similar energy. In addition, most of the participants of these studies were normal weight, thus it is less likely that a change in weight occurs following the intervention. On the other hand, consuming 25g of DC daily for 3 months (125 mg of polyphenols) resulted a slight increase in body weight (0.8 kg) ($p=0.03$) (Desch et al. 2010), a change that was not observed after the consumption of 6.3 g of DC containing 30 mg of polyphenols daily for 18 weeks (Taubert et al. 2007). Further human controlled intervention studies are essential to investigate the effect of DC on weight.

It is worth mentioning that it is not clear yet which amounts and type(s) of polyphenols / flavanols in cocoa/DC could decrease body weight/body fat in humans, as well as the mechanisms involved. In a mouse study, the administration of 2 mg/kg/day of DC-derived epicatechin improved aerobic metabolism and fat oxidation (Nogueira et al. 2011). In the 10-week study by Gu et al. (2013), which showed a favourable effect of cocoa on reducing obesity in mice, it was suggested that the dose of cocoa supplementation is equivalent to a daily amount of 54g of cocoa powder in humans (provides around 221.4 Kcal), which contains 24.84 mg of epicatchin and 513 mg of polyphenols. In addition to epicatechin, a study showed that procyanidins exert a higher obesity counteracting effect than epicatechin when these flavanols were administered to obesity-induced mice for a period of 12 weeks (Dorenkott et al. 2014). Quercetin was also attributed anti-obesity properties by

inhibiting adipogenesis in preadipocytes in vitro and causing apoptosis in mature adipocytes (Ahn et al. 2008).
Lastly, DC contains caffeine (Stark et al. 2006), which was previously presented as having a weight reducing effect (Zheng et al. 2004), thus it is important to closely match between the caffeine content of the control and the treatment products.

In view of the inhibitory effects of cocoa polyphenols on induced-obesity in animals and on adipose tissue differentiation in cell studies, it could be suggested that cocoa polyphenols are effective in counteracting obesity. However, the non significant change in body weight in humans renders the investigation of the effect of cocoa polyphenols in the context of a hypocaloric or normocaloric diet essential in order to identify the mechanism of action of these polyphenols, and whether they are only implicated in countering the gain in weight in individuals. Furthermore, it is important to determine whether the higher fat content of DC compared to cocoa can affect the weight counteracting properties of cocoa polyphenols, particularly that no animal studies have previously investigated the effect of DC on obesity.

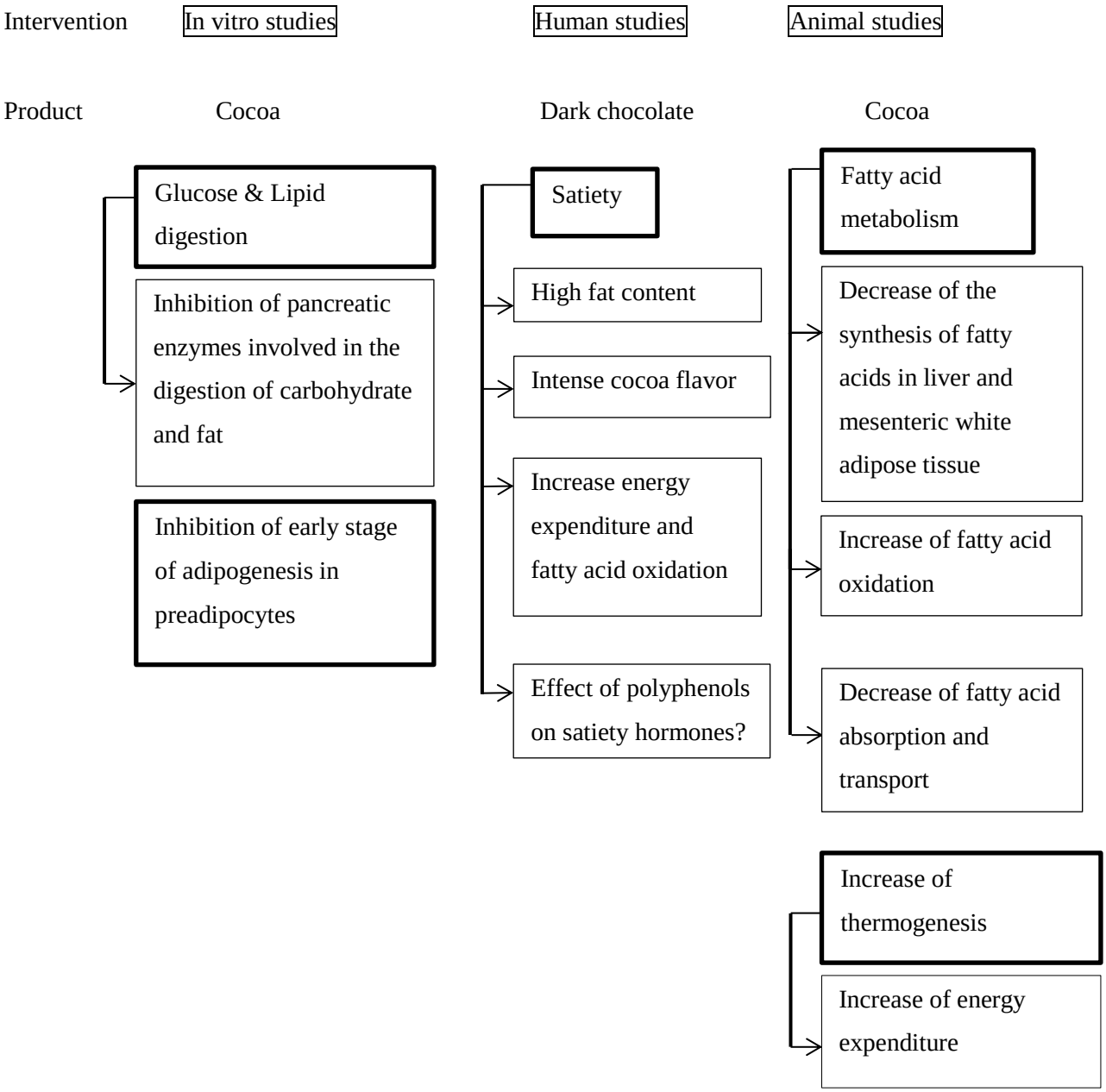



**Figure 1.20: Possible implications of cocoa/dark chocolate in body weight control**

## 1.10 Proposed investigations

The design of the proposed investigations was based on the following statements:

- Obesity and its complications are rising worldwide (CDC 2009a).
- Diet has been shown to have a significant impact on the prevention of obesity and associated chronic diseases (CDC 2009a).
- A small reduction in risk factors for type 2 diabetes and CVD has been associated with a substantial decrease in the occurrence of these diseases (Whelton et al. 2002; Mclnnes et al. 2005).
- Cocoa polyphenols have been largely studied for their favourable effects on risk factors for type 2 diabetes and CVD (Katz et al. 2011).
- Dark chocolate, a common snack, is a polyphenol-rich food (Bravo et al. 1998).

It was then hypothesized that a single dietary factor could exert a potential beneficial effect on health. Therefore, the aim of this research was to investigate the influence of PRDC (polyphenol-rich dark chocolate) on anthropometric, biochemical, nutritional and physiological markers in normal weight and overweight adults. For this purpose, two studies were carried out: the first study principally examined the effect PRDC on insulin sensitivity in normal weight and overweight adults, whereas the second study investigated the effect of PRDC on body weight in the adult overweight and obese population. A summary of the proposed studies is presented in table 1.9.

### 1.10.1 Study I: Effect of polyphenol-rich dark chocolate on insulin sensitivity in normal weight and overweight adults

#### 1.10.1.1 Rationale for study

There has been a considerable amount of research on the effects of polyphenols and dark chocolate on health, and their potential role in the modulation of risk factors for type 2 diabetes and CVD (Jia et al. 2010; Hooper et al. 2012; Ried et al. 2012). However, results are still conflicting. Thus, recognizing the potential benefits of DC on these risk factors is important to help the public make informed choices.

To our knowledge, only five human studies tested the effects of daily DC supplementation on insulin levels and insulin resistance/sensitivity in individuals with no diagnosed diabetes, hypertension and CVD (Grassi et al. 2005; Davison et al. 2008; Al Moosawi et al. 2012; Stote et al. 2012; Di Renzo et al. 2013). This limited number of studies is insufficient to identify an implication of PRDC in the prevention of IR and/or type 2 diabetes. As for serum lipids (TC, TG, HDL and LDL), few studies noted a favourable effect of PRDC on lipid profile in healthy individuals (Mursu et al. 2004; Baba et al. 2007b), while others (Mathur et al. 2002; Grassi et al. 2005) mostly showed no significant effect. Additionally, oxidative stress was linked to the occurrence of many chronic diseases (Urquiaga and Leighton 2000), and the limited studies showed an inverse association between PRDC consumption and oxidised LDL (Wan et al. 2001; Lettieri-Barbato et al. 2012). Hence, further studies are needed to elucidate this effect. Also, CVD were described as an inflammatory condition (Shah and Prediman 2009), and correlational studies found an inverse association between flavonoid/DC consumption and inflammation (Chun et al. 2008; Gisueppe et al. 2008). Thus, more intervention studies are needed particularly because of the discrepancy in the results between correlational and intervention studies (Intervention studies mostly showed no significant effect of DC on inflammation (Mathur et al. 2002; Allen et al. 2008; Nogueira et al. 2012). Testing hs-CRP levels was considered due to the fact that it is an important marker of inflammation, and a strong predictor of coronary artery disease (Jousilahti et al. 2001). Furthermore, few studies reported a decrease in blood pressure after the consumption of DC in normotensive individuals (Al Faris 2008; Al Moosawi et al. 2010), while many others showed no significant effects (Murphy et al. 2003; Engler et al. 2004).

Moreover, cortisol was shown to be implicated in IR, and the enzyme 11β-HSD 1 was responsible for increasing cortisol production and exacerbating obesity complications (Stulnig and Waldhausl 2004). As polyphenols were previously reported to lower cortisol excretion (Martin et al. 2009) and the activity of 11β-HSD 1 (Hindzpeter et al. 2014), measuring cortisol levels and the activity of 11β-HSD 1 (by the means of cortisol/cortisone ratio) would identify any potential decrease in this enzyme activity following the consumption of DC rich in

polyphenols. This is important because very few studies have been conducted on cocoa polyphenols and cortisol.
In addition, with the emerging animal and in vitro studies on cocoa/DC and their weight lowering properties (Matsui et al. 2005; Gu et al. 2012), it was aimed to test the effect of 4 weeks of PRDC supplementation on body weight and waist circumference in the context of a normocaloric diet.

In relation to baseline BMI status, it was shown in a previous study that normal weight and overweight individuals might respond differently to DC supplementation (Al Moosawi et al. 2012). Therefore, this study aimed to identify any potential difference in the response to intervention based on BMI status. This would be beneficial in possibly including this snack in future dietary strategies that aim to prevent further complications in the overweight and obese population. This study aims to investigate and possibly compare the results to the study held by Al Moosawi et al. (2012), in which the same type and daily amount of DC was used, and a lowering effect on HOMA-IR and blood pressure was only noted in overweight and obese individuals following PRDC consumption.
Lastly, recognizing the importance of prevention in reducing the prevalence of chronic conditions and improving the health and quality of life of nations (CDC 2011) was an important rationale for including participants with no hypertension, type 2 diabetes and CVD. Hence, this study might help in developing prevention strategies for reducing the incidence of these diseases.

#### 1.10.1.2 Aims and Objectives

The aim of this study was to assess the effect of PRDC on insulin sensitivity in normal weight and overweight adults.

**Objectives**

- To determine the effect of PRDC consumption on the following outcomes:

  -Primary outcome: Insulin sensitivity (determined by HOMA-IR and QUICKI)

-Secondary outcomes:

a. Glucose levels
b. Lipid profile (serum TC, HDL, LDL and TG levels)
c. Oxidised LDL levels
d. Hs-CRP levels
e. Blood Pressure
f. BMI and Waist circumference
g. Salivary cortisol levels
h. 11 β-HSD1 enzyme activity (salivary cortisol/cortisone ratio)

- To determine the impact of BMI (BMI between 18-24.9 kg/$m^2$ and over 25 kg/$m^2$) on the outcomes following PRDC consumption.

#### 1.10.1.3 Hypothesis

Regular consumption of PRDC might improve insulin sensitivity and LDL oxidation in the normal weight and overweight adult population.

### 1.10.2 Study 2: Effect of polyphenol-rich dark chocolate on body weight in overweight and obese adults

#### 1.10.2.1 Rationale for study

As little or no studies in the literature previously investigated the effect of 12 weeks of PRDC on body weight and composition, testing the feasibility of the protocol by doing a small scale study was crucial, as suggested by Arnold et al. (2009). This was mainly to provide preliminary results for a larger study, and information about the validity of the proposed study design (including frequency of appointments) and the suggested methods (including questionnaires (food frequency questionnaire, physical activity questionnaire) and inclusion and exclusion criteria) (Thabane et al. 2010). Finally, results of this study were intended to be used to perform sample size calculations for a large trial.

The investigation of the effect of PRDC rich in polyphenols on body weight was based on: 1) Polyphenols, and particularly cocoa polyphenols, were recently

involved in the modification of molecular and physiological pathways implicated in adiposity and energy metabolism (Matsui et al. 2005; Yamashita et al. 2013). 2) DC is a high source of polyphenols (McShea et al. 2008). 3) Diet and exercise were shown to be effective in the treatment and prevention of obesity (Glanville et al. 1997; NHS 2012b). However, the lack of willpower and the failure to maintain the weight loss for a long term (Foster et al. 2005; Weiss et al. 2007) rendered these strategies sometimes inefficient in treating or preventing obesity. Therefore, complementary approaches might exert a significant implication in weight management. 4) Even a modest loss in weight (5-10 %) was associated with a decrease in risk factors for diabetes type 2 and CVD (Resnick et al. 2000; Wing et al. 2011). Consequently, the identification of weight lowering properties of PRDC could possibly help in decreasing risk factors for chronic diseases.

The effect of cocoa polyphenols on counteracting obesity was previously reported by several animal studies which documented an inhibitory effect of cocoa on fatty acid gene synthesis (Matsui et al. 2005; Gu et al. 2013; Min et al. 2013). In addition, in vitro studies reported an effect of cocoa extracts on the inhibition of digestive enzymes involved in the hydrolysis of carbohydrates and lipids (Gu et al. 2011). These effects have not been yet evidenced by human intervention studies. Also, an epidemiological study found an inverse association between chocolate consumption and BMI in 1018 adults with no known diseases (Golomb 2012), and highlighted the need for randomized trials to assess the relationship between cocoa polyphenols and body weight. As for waist circumference, it was shown to be decreased after one week of daily DC supplementation ($-1.24 \pm 1.45$ cm, $p \leq 0.05$) in 15 adult women (Di Renzo et al. 2013). Hence, it was important to test the long term effect of PRDC on WC.

Furthermore, an increase in basic metabolic rate (BMR) (by 42 Kcal/22h, $p < 0.05$) was noted in mice fed flavanol-derived cocoa for 2 weeks (Osakabe 2013), while another study showed an increase in lean muscle mass and oxidative phosphorylation in mice administered epicatechin-derived cocoa (Nogueira et al. 2011). Hence, estimating energy expenditure and body composition might provide an explanation

for any potential changes in anthropometric measures and energy expenditure resulting from an increase in lean muscle mass.

This study aimed to investigate the effect of DC supplementation on body weight when incorporated into a regular diet, and not as a part of a reduced Calorie diet or in addition to exercise. This intended to identify whether the lowering effect on body weight- if occurred- is due to the consumption of a sweet snack which enhanced diet satisfaction (Piehowski et al. 2011) (in this case, weight might decrease in both placebo and PRDC groups), or to the implication of polyphenols, but not to the effect of a hypocaloric diet. Thus, a polyphenol-rich snack was compared against a low-polyphenol snack to test if PRDC produces unique effects when incorporated into a normocaloric diet. This might provide evidence for testing the implication of PRDC in weight loss regimens.
Furthermore, the study of Al Mossawi et al. (2012) showed an increase in BMI following placebo DC consumption (which had a similar macronutrient composition to the placebo DC used in this study). Therefore, it was important to test the detrimental effect of a low-polyphenol chocolate on body weight when administered for a long term (12 weeks).

Since oxidative stress was related to the occurrence of many diseases such as hypertension and CVD (Urquiaga and Leighton 2000), identifying the antioxidant properties of DC was considered. This was examined by analysing total polyphenols in the urine (through the Folin-Ciocalteu method), and total antioxidant capacity (through the ferric-reducing antioxidant power (FRAP) method). The latter two parameters were also used as biomarkers of compliance to intervention.
As for glucocorticoid metabolism, cortisol was related to obesity complications, and was reported to increase food and energy intake (Dallman et al. 2004), and to induce IR via inducing gluconeogenesis. In addition, the enzyme 11β-HSD 1 is highly expressed in the adipose tissue of obese individuals, and is responsible for increasing cortisol production and exacerbating obesity complications (Stulnig and Waldhausl 2004). As polyphenols were reported to lower cortisol (Martin et al. 2009) and 11β-HSD 1 activity (Hindzpeter et al. 2014), measuring cortisol levels and the activity of

11β-HSD 1 in the urine was considered to identify any significant change in these parameters following the consumption of DC rich in polyphenols.

Furthermore, the supplementation of a single dose of DC was linked to a decrease in appetite (Sorenson and Astrup 2011). Hence, obtaining qualitative data on the long term changes in appetite will provide rationale for studies to test the effect of PRDC on satiety hormones (such as cholecystokinin and PYY). Lastly, the concept of whether exposure to food temptations (sweet snack) results in a healthier food (Kroese et al. 2009) was tested through looking at changes in the intake of sugar and saturated fatty acids throughout the intervention.

This study also aimed to look at the practicality of the daily long term (12 weeks) consumption of 20g of DC daily. As some complaints regarding chocolate consumption in studies lasting from 2-8 weeks were noted (Ried et al. 2012), it was important to test the long term side effects of chocolate consumption on a small number of participants. This might help making suitable recommendations for future large studies.

#### 1.10.2.2 Aims and Objectives

The overall aim of this study was to determine the effect of PRDC on body weight and overweight and obese adults.

a. Scientific objectives

a.1. To determine the effect of long term PRDC supplementation on the following outcomes:

Primary outcome:

- Body Weight

Secondary outcomes:

- Body Fat percentage
- Waist circumference
- Cortisol levels in the urine
- Activity of 11 β-HSD1 (urinary cortisol-to-cortisone ratio)
- Basic metabolic rate (BMR)
- Lean body mass (LBM)
- Total polyphenols in the urine (determined via Folin-Ciocalteu method)
- Total antioxidant capacity in the urine (determined via FRAP assay)

a.2. To determine the effect of placebo DC supplementation on the primary and secondary outcomes

b. Feasibility objectives

b.1. To determine the practicability of the study procedures by assessing:

- Study design
- Drop out rates
- Compliance rates
- Data collection tools and questionnaires
- Side effects of placebo / treatment

b.2. To determine the sample size needed to conduct a large trial based on the primary outcome measure.

### 1.10.2.3 Hypothesis

Long term consumption of dark chocolate (12 weeks) may reduce body weight in the adult overweight and obese population

**Table 1.9: Summary of the proposed studies**

| | Arms | Design | Duration | Primary outcome |
|---|---|---|---|---|
| Study I | Two arms :<br>• DC rich in polyphenols (500 mg)<br>• DC with low amount of polyphenols (placebo) | Parallel placebo-controlled Trial | 4 weeks | Insulin sensitivity (determined by HOMA-IR and QUICKI) |
| Study II | Two arms :<br>• DC rich in polyphenols (500 mg)<br>• DC with low amount of polyphenols (placebo) | Parallel placebo-controlled trial | 16 weeks | Body weight (BMI) |

# CHAPTER 2: GENERAL MATERIALS AND METHODS

## 2.1 Ethical approval

The ethical approval for the two studies was granted by Queen Margaret University ethics committee. The studies were performed according to the declaration of Helsinki (WMA 2013). Participants were provided a written information sheet, and were asked to sign a written consent form before taking part. The first and second studies were registered on *clinicaltrials.gov* as NCT01749020 and NCT01749852, respectively. Handling of data was managed according to the Data Protection Act (1998) (Legislation 1998).

## 2.2 Nutritional and chemical composition of the experimental dark chocolate

A summary of the nutritional and chemical composition of the placebo DC and the PRDC used in both research studies is presented in Table 2.1. The products were provided free of charge by Barry-Callebaut Company, Belgium. The amount of 20g is the daily portion that was given to participants in both trials. PRDC (500 mg of polyphenols) was produced via a method called "Acticoa" - trademark of Barry Callebaut-, which aims to naturally preserve polyphenols in different steps of the manufacturing chain (Barry Callebaut 2010). The two types of chocolate looked identical, and contained 63.5% of cocoa solids. In order to avoid confounding factors, the two dark chocolate types (placebo and control) were fairly matched for macronutrient and micronutrient content, and contained similar amounts of caffeine and theobromine. Thus, they only differed by polyphenol content.

To validate the data provided by the company, the analysis of flavanols for the two types of chocolate was also performed at the "James Hutton institute", Dundee (Dr Gordon Mcdougall). The LC-MS (Liquid chromatography–mass spectrometry) method was carried out to determine the total flavanol content and flavanol fractions in both chocolate types. The content of theobromine and caffeine was also determined using LCMS.

For this method, extraction of samples was required. The chocolate samples were stored at -20$^{o}$C, then grounded to a powder in an electric coffee mill in a cold room

(< 5$^{o}$ C). Three samples of 0.5g of each of the two types of chocolate were subsequently weighed into labelled tubes. These samples were then extracted with 5 ml of 50 % acetonitrile/ultra pure water containing 0.2 % (v/v) formic acid, vortex mixed (3 times for 15 seconds each, full speed), and sonicated for 5 minutes in ice-cold water. After centrifugation (10 mins, 4000 rpm, 5 $^{o}$C), the supernatants were removed to fresh tubes and the pellets re-extracted with further 7.5 ml of 50 % acetonitrile/ultra pure water containing 0.2 % (v/v) formic acid. Samples (1 ml) were then dried in a speed vac by rotary evaporation, resuspended in 5 % acetonitrile before being subjected to the LC-MS method.

An LCQ-DECA system, comprising a Surveyor auto-sampler, a pump, a photo diode array detector (PDAD) and a Thermo Finnigan mass spectrometer ion trap was used for LC-MS. Solvent A was ultra-pure water containing 0.1% formic acid (v/v), and solvent B was acetonitrile containing 0.1 % formic acid (v/v). 20 µl of each sample were eluted on a gradient of: t= 0-5mins, 0% B; 5-35 minutes, 0–40 % B, followed by a wash to 100% B with the gradient returning to the starting conditions at 50 minutes. A C18 column (Synergi HydroC18 with polar end capping, 2 mm X 150 mm, Phenomenex Ltd.) was used at 200µl/min, and the three discrete channels were scanned by the PDAD (280, 365 and 520 nm). An electrospray ionisation interface was fitted to the LCQ-DECA LC–MS, and the samples were analysed in positive and negative modes. There were 2 scan events: full scan analysis, followed by data dependent MS2 of the most intense ions using collision energies (source voltage) of 35 % in wideband activation mode. Catechin and cyanidin-3-O-glucoside were used to tune the MS detector for negative and positive modes respectively. Components were identified using literature data (Gu et al. 2006), and their content was assessed against epicatechin. The differences in the amounts of flavanols and methylxanthines between placebo DC and PRDC are illustrated in Figure 2.1, and the quantification of the amount of flavanols is presented in Table 2.2. The results fairly matched with the company's data and confirmed the elimination of the possible confounding factors of caffeine and theobromine.

**Table 2.1: Nutritional and chemical composition of the experimental dark chocolate**

| **Components** (per 20g daily portion) | **PRDC** | **Placebo DC** |
|---|---|---|
| Energy (kcal) | 102 | 102 |
| Total fat (g) | 7.34 | 7.34 |
| Carbohydrates (g) | 7.44 | 7.44 |
| Protein (g) | 1.34 | 1.34 |
| Total flavanols (mg) | 400 | < 60 |
| Epicatechin (mg) | 85 | 12 |
| Catechin (mg) | 15 | 2 |
| Caffeine (mg) | 15 | 15 |
| Theobromine (mg) | 150 | 150 |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Data obtained from Barry-Callebaut company, Belgium. Total flavanols, epicatechin and catechin were analysed via LCMS method (Liquid chromatography–mass spectrometry). The amount of total polyphenols in the PRDC (500 mg) was assessed by Folin-Ciocalteu method, as provided by the company.The amount of 20g is the daily amount provided to participants in both trials.*

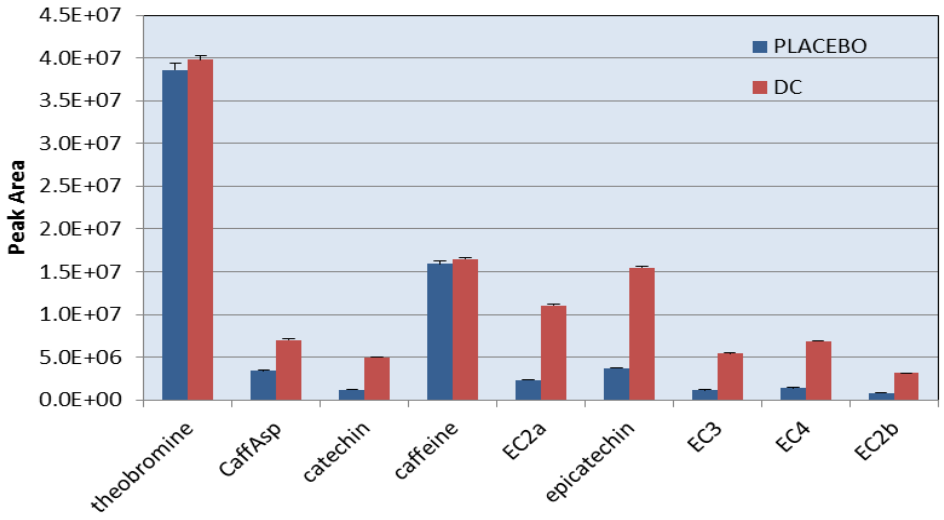



**Figure 2.1: Differences in polyphenol and methylxanthine contents between placebo DC and PRDC**

*Caff Asp: N-caffeoyl-l-aspartate, EC: Epicatechin. DC (dark chocolate) represents the PRDC (Polyphenol-rich dark chocolate) and placebo represents the placebo DC. Samples were analysed at James Hutton institute via LCMS (Liquid chromatography–mass spectrometry) and quantified by peak area. EC2a, EC2b, EC3, and EC4 represent the number of EC units– EC2a and b are epicatechin isomers.*

Table 2.2: Quantification of flavanols in the placebo DC and the PRDC

| Component | Content (mg of epicatehin equivalents/20g portion) | | | |
|---|---|---|---|---|
| | PLACEBO | PRDC | ±SE | ±SE |
| Catechin | 7.04 | 34.67 | 0.17 | 0.42 |
| EC2a | 14.10 | 76.87 | 0.34 | 0.94 |
| EC2b | 5.56 | 21.61 | 0.12 | 0.26 |
| Epicatechin | 21.35 | 107.27 | 0.54 | 1.31 |
| EC3 | 7.04 | 37.57 | 0.17 | 0.46 |
| EC4 | 7.01 | 47.52 | 0.20 | 0.58 |
| EC5 and greater* | 5.43 | 69.13 | 0.15 | 0.09 |
| TOTAL mg of flavanols /20g portion | **67.53** | **394.64** | **0.24** | **0.58** |

**The estimation of the contribution of the larger components might be inexact. EC: epicatechin, PRDC: Polyphenol-rich dark chocolate. Samples were analysed at James Hutton institute via LCMS (Liquid chromatography–mass spectrometry). EC2a, EC2b, EC3, and EC4 represent the number of epicatechin units. EC2a and b are epicatechin isomers. The values are averages of three determinations and are expressed in mg of epicatehin equivalents ± standard error (SE). The amount is per 20g portion.*

## 2.3 STUDY I

### 2.3.1 Study population

#### 2.3.1.1 Recruitment and selection of participants

Volunteers were recruited via several means: QMU University Moderator (emails sent to all staff and students at QMU), flyers and posters in community and sports centres in Edinburgh and Musselburgh, hospitals in Edinburgh, colleges and universities in Edinburgh, as well as via word-of-mouth.

**Inclusion and exclusion criteria**

Participants were recruited and studied with no restriction to race or socioeconomic status according to the following criteria:

***Inclusion criteria***

-Adults with no history of hypertension, type 2 diabetes or CVD.

-BMI between "18-24.9 kg/$m^2$" and BMI between "25-34.9 kg/$m^2$".

- Males and Females

-Age: 18-65 years

***Exclusion criteria***

-Participants with CVD, hypertension or diabetes

-Participants taking medications that affect insulin, glucose, lipid, hs-CRP and/or blood pressure levels

-Participants taking dietary supplements containing high doses of antioxidants

-Postmenopausal women taking HRT (Hormone Replacement Therapy)

-Participants who recently participated or are currently on a weight management program

-Smokers and heavy alcohol drinkers (defined as more than 15 drinks/week for men and more than 8 drinks for women (CDC 2014)).

-Participants with regular consumption of cocoa or DC (> 1 serving/week) (Taubert et al. 2007).

### 2.3.2 Determination of sample size

The proposed analysis is an ANCOVA. Therefore, for sample size estimation, the calculation followed that of Borm et al. (1997) where we first calculate the sample required for an independent t-test and then modify this accordingly; recognising that given a pre-post design, there is less error. The assumed pre-post correlation required for the second part of the calculation was set at 0.6, which seems a reasonable assumption. Therefore, a sample size of 45 in each group with 80% power and using a two group t-test with a 0.05 two-sided significance level was needed to detect an effect size of 0.6. Adjusting for the non-independence of the pre-post calculations with an assumed pre – post correlation of 0.6, the number required for this given effect size was reduced to 30 per group, or 60 in total. With an estimated attrition of approximately 15 to 20%, the aim was to recruit 74 persons in total. Although the

original purpose was to determine the sample size based on the results of the study undertaken by Al Moosawi et al. (2012), which showed that PRDC containing 500 mg of polyphenols reduces HOMA-IR in female adults, this was not possible due to the fact that all data were stratified between normal weight and overweight participants. However, the principal objective of this study was to determine the effect of PRDC on HOMA-IR in the general population. Stratifying participants according to weight status was indeed a secondary outcome. In addition, the original data from the latter study was 1discarded upon completion of the study due to ethical considerations. Hence, the use of these data was not possible at this stage, and the determination of sample size was carried out via the above method.

### 2.3.3 Study design

A feasibility study (including 6 participants) was first carried out to ensure the possibility of carrying out the intervention with the current experimental design. All measurements were taken, and the intervention lasted for 4 weeks. The data collected from the six participants were then added to the main study.
The main study is a randomized single-blinded placebo controlled pre-post intervention parallel study with 2 arms where participants received one of the 2 different types of DC according to the following:

Type A: Placebo DC (low in polyphenols)
Type B: DC rich in polyphenols (500 mg)

Randomization was implemented using sealed envelopes based on the protocol described by Doig and Simpson (2005). Participants were stratified on one factor (BMI). Seventy-four standard-sized sheets were marked by either P (Placebo)-37 sheets or D (PRDC)-37 sheets, coupled with O: Overweight or N: Normal weight. Sheets were labelled by codes (PN1-PN19; PO1-PO18; DN1-DN19 and DO1-DO18). The codes PO1-PO18 and DO1-DO18 were placed in the “overweight strata” container, and the codes PN1-PN19 and DN1-DN19 were placed in the “normal weight strata” container. The sheets were inserted into unlabelled envelopes and sealed. Then, the researcher chose an envelope from the overweight strata

container if the participant was overweight, or an envelope from the normal weight strata container if the participant was normal weight.

Participants were provided an information sheet and asked to sign a consent form (Appendix 1) before the beginning of the study. Volunteers were then requested to visit Queen Margaret University clinical Lab, where the study was held. Each of the two appointments lasted for 25-30 minutes (View Table 2.3 for the timeline of the appointment). Participants randomly received 20g of DC daily (Type A or B) for a period of 4 weeks. Blood and saliva samples, blood pressure and anthropometric measurements were taken before the start and at the end of the intervention (after 4 weeks). A general questionnaire (Appendix 2) and questionnaires related to physical activity (Appendix 3) and acceptability (Appendix 4) were requested to be filled. Participants were asked to maintain their usual diet and physical activity throughout the intervention period. A diet adjustment was also considered so that DC substitutes another food of similar energy. The researcher helped in providing dietary advice for participants to help them achieve this goal. This happened by asking participants about the types of snacks usually consumed. Suggestions for replacing a part of the snack or the overall snack by the experimental DC were then provided. To monitor changes in dietary intake, participants were asked to fill a 3-day diet diary (Appendix 5) at baseline and during the third week (View Figure 2.2 on study design) (The details of collected data are presented in section 3.3.5).

Before attending the appointment, participants were asked to:

-Be on a fasting state (which is defined as the restriction from eating or drinking any liquids for a period of 8 hours (Diabetes UK 2013).

-Avoid drinking alcohol for the past 24 hours

-Consume the last dose of DC at least 12 hours before the second appointment

-Refrain from doing a high level of physical activity at least 12 hours prior to the visit

-Avoid or reduce eating high quantities of foods rich in flavonoids (>4 mg/100g of selected foods, knowing that a medium content of flavonoids in foods is between 4-9.9 mg/100g and a high content constitutes more than 10mg/100g of selected foods (O'byrne et al. 2002)) one week before the start (run-in phase) and subsequently for

the duration of the study. Examples include red wine, blackberry, blueberries, tea as well as dark chocolate and cocoa products (View Appendix 6 for the list of foods rich in flavonoids that was provided to participants).

**Table 2.3: Timeline for Study I appointments**

| | Minutes |
|---|---|
| Introduction | 3 |
| Anthropometric measures | 5 |
| Blood Pressure | 11 |
| Blood withdrawal | 3-8 |
| Questions | 3 |
| Total | 25-30 |

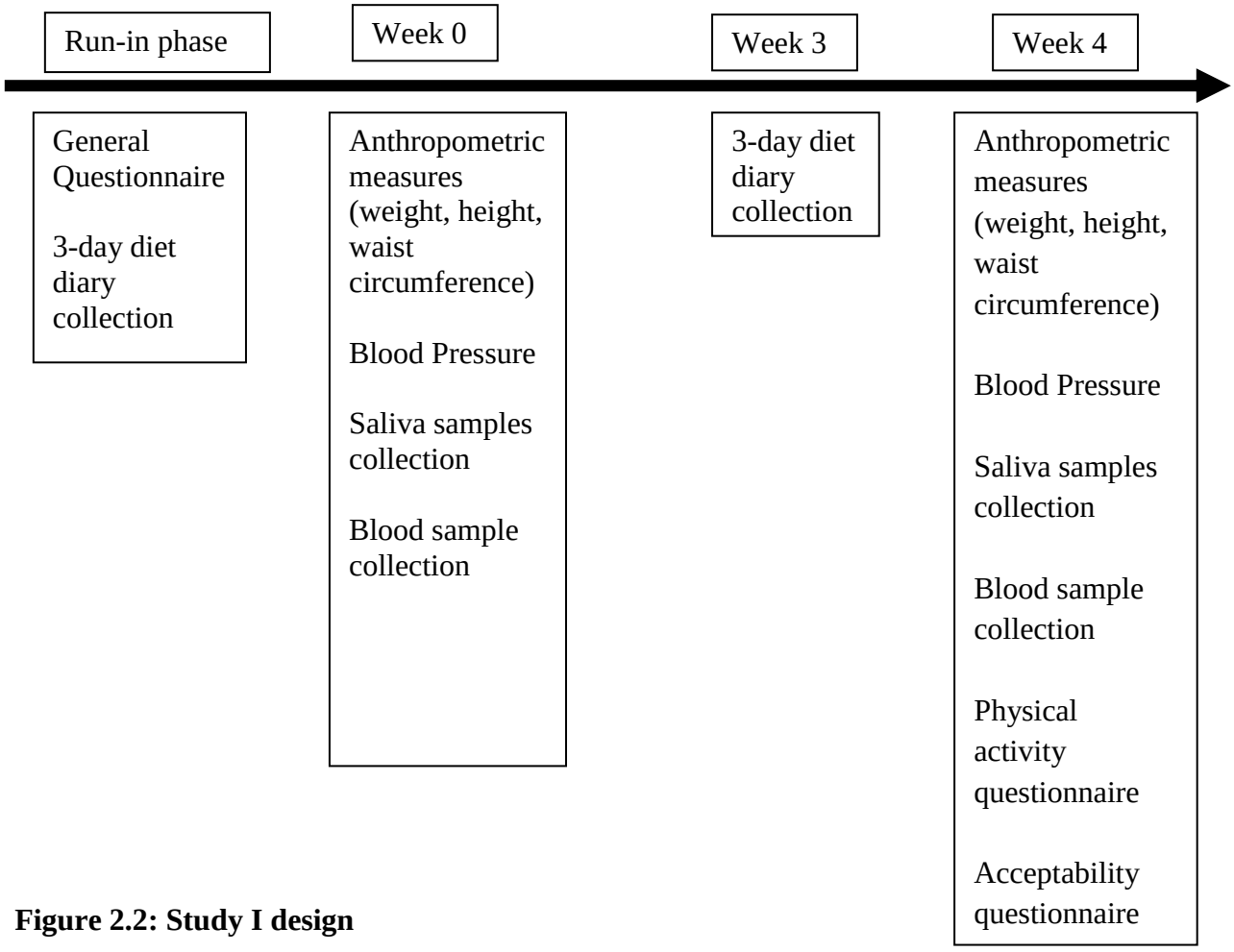



**Figure 2.2: Study I design**

### 2.3.4 Justification for the chosen methods

#### 2.3.4.1 Study duration

The choice of the study duration was primarily based on the previous study held by Al Moosawi et al (2012), which reported a significant decrease in HOMA-IR following four weeks of daily consumption of PRDC (500 mg of polyphenols). In addition, Di Renzo et al. (2013) and Stote et al. (2007) did not note a decrease in HOMA-IR after 5-7 days of PRDC supplementation with doses of polyphenols varying between 22-900 mg of flavanols. It was therefore suggested that a longer duration is needed to induce an effect on insulin metabolism. Although the study of Grassi et al. (2005) showed an improvement in HOMA-IR after 2 weeks of daily dark chocolate consumption (with 500 mg of polyphenols), testing the effect of PRDC on insulin responses over a longer period was aimed. This was particularly important to identify the possibility of an adaptation effect to the quantity of polyphenols over time, which was previously suggested (Al Moosawi et al. 2012). Additionally, it was reported that an adequate time to detect potential changes in different markers is needed. For instance, serum lipid levels become stable after a 3-week dietary intervention. Consequently, a 4-week study might be suitable to increase reliability of end point measurements (Monsen and Van Horn 2007).

#### 2.3.4.2 Study design

The choice of a parallel design was based on the advantages of this design compared to a crossover design. In parallel studies, participants are allocated one study treatment for the whole period of study, whereas in crossover trials, participants are assigned all the treatments of the study. Several factors may affect the choice of study design (like the objective and duration of the study, the length of washout periods, the time needed to induce an effect, and the number of treatments) (Monsen and Van Horn 2007). Parallel studies have the potential advantage of convenience due to the shorter overall study duration, which results in a lower dropout rate and a higher adherence to study protocol compared to crossover trials. In addition, the analysis is more straightforward, and the precision of this design can be enhanced by the use of statistical adjustments for baseline measurements (Bailar and Loaglin

2009). Furthermore, a parallel design allows the comparison between different dose levels of a treatment in one study (Richens 2001). Moreover, the absence of a carry-over effect and an order effect (defined by an influence of the first treatment on the response to the second treatment (Richens 2001)) are the main advantages of parallel trials compared to crossover trials (Monsen and Van Horn 2007). Nonetheless, it is important to be aware of the disadvantages of a parallel design, like the need for a high number of participants (Bailar and Loaglin 2009), and the increase in interparticipant variation (since the participant does not serve as his own control) (Monsen and Van Horn 2007). These limitations constitute the reasons why crossover studies can be considered more powerful than parallel studies (Bailar and Loaglin 2009). However, although both parallel and crossover trials might be subject to expectation bias resulting in a positive response to placebo (placebo effect) or a negative response to treatment (nocebo effect), there is some evidence to suggest that due to a carry-over effect, crossover studies are unblinded and therefore, result in a lower placebo effect and a higher treatment effect than parallel trials (Feys et al. 2012).

Therefore, the decision of adopting a parallel design was mainly based on the absence of carry-over and order effects. Additionally, the possible length of the washout period required to minimize the carry-over effect of polyphenols was estimated to be around 2 weeks in some studies (Grassi et al. 2005; Al Moosawi et al. 2010; De Bock 2013). This duration, along with the study period (4 weeks) made it more convenient to opt for a parallel design in order to maintain participants' commitment and decrease attrition rates (Dash et al. 2012).

#### 2.3.4.3 Quantity and polyphenol content of DC

The quantity of DC (20g) was selected as it is a small quantity, commonly considered less than a serving of dark chocolate (a serving of DC is considered to be around 32g (ADA 2007), or between 25-50g (Lewis et al. 2010)), and providing around 102 Kcal. This amount of energy contributes to no more than 6% of the daily women's intake and 4% of the daily men's intake (CDC 2011b). In addition, a recent study aiming to quantify a moderate daily candy intake, showed that 50-100 calories per day constitute a reasonable intake of candy. This amount is based on the

recommendations of health authorities for fat, sugar and calorie intake, and varies depending on energy needs (Hornick et al. 2014). Consequently, the energy content in the experimental DC study was not supposed to cause major increases in daily energy intake in both genders.

The choice of the dose of polyphenols (500 mg) was based on its ability to improve insulin sensitivity in the studies of Grassi et al. (2005) and Al Moosawi et al. (2012). Also, many studies discussed the threshold effect of 500 mg of polyphenols (Al Moosawi et al. 2010; Shrime et al. 2011; Tokede et al. 2011), as well as the potential side effects (such as gastrointestinal discomfort and nausea (Chow et al. 2003; Mead 2007)) and the decrease in palatability (Al Moosawi et al. 2010) resulting from a higher polyphenol dose.

### 2.3.5 Data collection and processing

#### 2.3.5.1 Questionnaires

*a. General Questionnaire*

Participants answered a brief questionnaire which covered information on social characteristics, lifestyle habits and medical history (Appendix 2). This was mainly designed to understand the characteristics of the population, and to ensure all participants complied with the inclusion criteria, explained in detail in the information sheet.

*b. Physical activity questionnaire*

This short questionnaire was filled twice during the intervention to identify whether participants maintained the same level of physical activity during the study period (Appendix 3). The baseline physical activity questions were included in the general questionnaire and filled prior the first appointment. Hours of physical activity were then transformed into MET (Metabolic equivalent task) units/week. MET is defined as the ratio of the metabolic rate of the exercise to the resting metabolic rate. It is postulated that one MET corresponds to the energy when seated at rest. MET is mainly calculated to evaluate the actual physical activity based on its energy expenditure, particularly that individuals engage in different types of exercise

activities throughout the week (USC 2003). Therefore, assigning a value for each type of physical activity based on its intensity will allow the comparison between physical activity levels of participants. Each hour of reported physical activity was quantified into MET per hour based on the type of activity (Table 2.4), and the sum of weekly hours was considered to determine METs/week.

**Table 2.4: Metabolic equivalent tasks for common physical activities**

| **Activity** | | **MET/hour** |
|---|---|---|
| Walking | Slowly (1.86 mph) | 1.8 |
| | Briskly (3.11 mph) | 3.2 |
| | Briskly (4.4 mph) | 5.3 |
| Jogging | 5.6 mph | 8.8 |
| | 6.8 mph | 11.2 |
| Running | 8 mph | 12.9 |
| | 9.32 mph | 14.6 |
| Badminton | | 3 – 4 |
| Aerobic dancing | Low | 3.9 |
| | Medium | 6.0 |
| Ballet dancing | | 6 – 8 |
| Yoga | | 3.2 |
| Basketball | | 11.1 |
| Bicycling | | 4.8 |
| Football | | 6-7 |
| Volleyball | | 6 |
| Hiking | | 6 |
| Horse riding | | 3.2 - 8.6 |
| Weightlifting | | 3 – 7 |
| Rugby | | 12.6 |
| Tennis | | 6 – 8 |
| Swimming | | 6.8 |

(Adapted from Jette et al. 1990)

*MET: Metabolic equivalent task; Mph: miles per hour. MET values are per hour of physical activity*

*c. Acceptability questionnaire*

Acceptability questionnaire was designed to obtain qualitative data from participants on whether they found it acceptable to consume the dose of dark chocolate daily, and if they will keep consuming the chocolate frequently following the intervention (Appendix 4).

2.3.5.2 Anthropometry

Anthropometric measurements were taken at baseline and week 4, and included weight, height, and waist circumference.

*a. Height*

Height was measured with person bare foot using a stadiometer. Participants were asked to have minimal clothes so that the posture is clear, and to stand in a straight position, the head being in the Frankfurt plane, and the palms facing the thighs. Volunteers were requested to have their knees straight and their feet together, with their shoulder blades, heels and buttocks vertically in contact with the stadiometer (Gibson 2005) (Figure 2.3). Measurements were taken twice and repeated if they differ by more than 0.5 cm (Daniels et al. 2000).

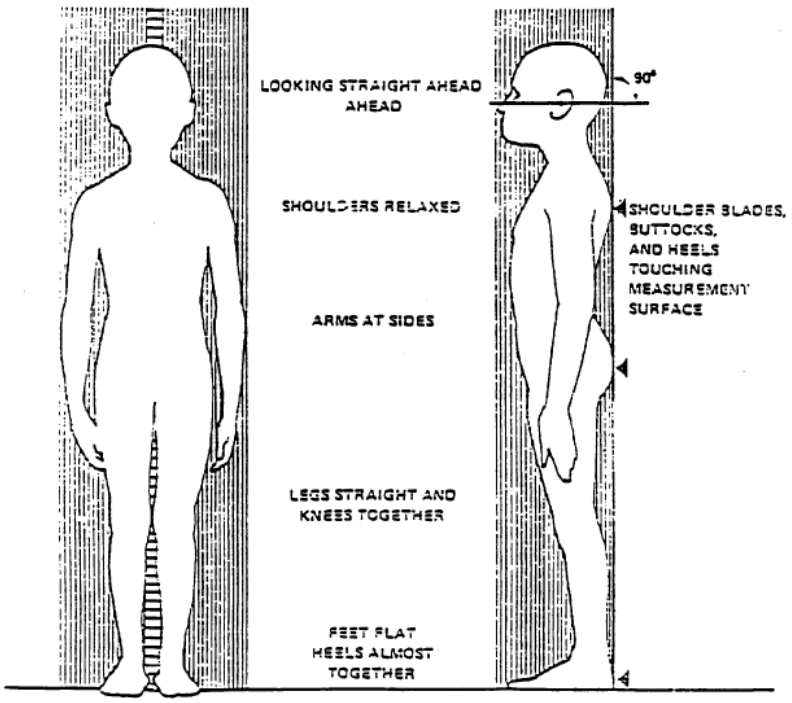



**Figure 2.3: Position of the body during height measurement**
(CDC 1988)

*b. Weight*

Weight was measured in the morning at fasting using an electronic scale (Tanita BF-559, Body Fat Monitor/Scale) positioned on a flat surface. Participants were asked to stand on the scale barefoot and with light clothing (Gibson 2005). Measurements were taken twice and repeated a third time if the first 2 measurements differed by more 0.3 Kg (Daniels et al. 2000).

The body mass index (BMI) was then calculated:

$$\text{Body Mass Index} = \frac{\text{Weight}}{(\text{Height})^2}$$

$$(\text{Kg}/\text{m}^2)$$

Also known as Quetelet index, BMI is commonly used to determine weight status in adults (Gibson 2005), and its relationship with the occurrence of diseases (Table 2.5). BMI, CVD risk factors and CVD increase in a linear manner (Lamon-Fava et al. 1996). The advantages and drawbacks of BMI have been discussed in section 1.1.2.1.

**Table 2.5: Relationship between BMI and disease risk**

| Classification | BMI Category ($kg/m^2$) | Risk of developing health problems |
|---|---|---|
| Underweight | < 18.5 | Increased |
| Normal Weight | 18.5 - 24.9 | Least |
| Overweight | 25.0 - 29.9 | Increased |
| Obese class I | 30.0 - 34.9 | High |
| Obese class II | 35.0 - 39.9 | Very high |
| Obese class III | >= 40.0 | Extremely high |

(Gibson 2005)

*c. Waist circumference*

Waist circumference was measured via a metal measuring tape, and was placed around the waist at the middle point between the lowest rib and the top of the hip bone. The tape was snug (without compressing the skin) and parallel to the floor (WHO 2008). For some obese participants with whom it was difficult to find the waist narrowing, the measurement was taken at the umbilical level, as described by Lohman et al. (1988).

Participants were asked to stand with arms at the sides, and to put their feet together. Measurements were taken twice after normal expiration, based on the protocol described in the WHO report, and repeated if there is a difference of more than 1cm between the 2 values (WHO 2008).

WC is an important predictor of disease risk, and is strongly correlated to obesity-related complications (Rexrode et a. 1998). Studies have shown that BMI and WC are two independent predictors of mortality risk, and they exert separate effects on the complications of morbidity (Janssen et al. 2004). Hence, for a similar BMI, individuals with a higher WC are at higher risk of diseases. In addition, individuals with a normal BMI and a high WC are at increased disease risk (NHLBI 2000). Measuring BMI and WC in this study allowed the analysis of a linear relationship between the two parameters. Also, WC measurement helped in overcoming the drawbacks of BMI in assessing all individuals with a high risk of metabolic complications.

Although some studies discussed the importance of waist-to-hip ratio (WHR) in predicting cardiovascular risk (De Koning et al. 2007) and type 2 diabetes risk (Qiao and Nyamdorj 2009), other studies showed that WC is more correlated with abdominal fat and total fat than WHR (Daniels et al. 2000). Also, WC is more associated with visceral obesity-associated complications than WHR, the latter being recognized as the pear-shaped obesity type (Cheng 2005). This made WC a better choice in research settings, and the sole instrument for measuring waist in this study. The cut-offs for WC were first defined to be ≥102cm in men and ≥88cm in women (WHO 2008). The International diabetes federation, in a position statement about the metabolic syndrome, lowered the ranges of WC, and adapted them to several

ethnic groups (IDF 2005) (Figure 2.4). This definition was shown to be more sensible in predicting cardiovascular risk (IDF 2005), and was considered by this study to determine individuals at high metabolic risk.

| Country/Ethnic group | | Waist circumference |
|---|---|---|
| **Europids*** In the USA, the ATP III values (102 cm male; 88 cm female) are likely to continue to be used for clinical purposes | Male | ≥ 94 cm |
| | Female | ≥ 80 cm |
| **South Asians** Based on a Chinese, Malay and Asian-Indian population | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| **Chinese** | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| **Japanese**** | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| **Ethnic South and Central Americans** | Use South Asian recommendations until more specific data are available | |
| **Sub-Saharan Africans** | Use European data until more specific data are available | |
| **Eastern Mediterranean and Middle East (Arab) populations** | Use European data until more specific data are available | |

** In future epidemiological studies of populations of Europid origin, prevalence should be given using both European and North American cut-points to allow better comparisons.*
*** Originally different values were proposed for Japanese people but new data support the use of the values shown above.*

**Figure 2.4: Classification of waist circumference based on ethnicity**
(IDF 2005). *ATP III: Adult treatment panel III.*

### 2.3.5.3 Blood pressure

Blood pressure was measured using a digital sphygmomanometer (Omron MS-1), with the participants in a seated position, after resting for 10 minutes. For the sake of standardization, blood pressure was measured using the right arm after being rested, based on the CDC recommendations. The arm was positioned in a way that the midpoint of the upper arm was as the same level as the heart (CDC 2009b), and the cuff was placed 2-3 cm above the pulsation of the brachial artery (O'Brien et al. 1997).

Measurements were taken three times and the average was considered. The classification of blood pressure was based on the NICE classification for blood pressure (Classification cited in Table 1.2).

#### 2.3.5.4 Haematological tests

*a. Venepuncture and processing of serum samples*

Samples of blood were collected from the anticubital vein with minimal stasis by a butterfly needle (Vacuette®, Greiner bio-one), using 3 vacutainers of EDTA (Ethylene Diamine Tetra Acetic Acid) (9 ml), Sodium Heparin (9 ml) and Sodium Fluoride/Potassium Oxalate (2 ml) (Vacuette®, Greiner bio-one). The blood sample drawn at each time was around or less than 20 ml. The samples were then ringed, and immediately centrifuged for 10 min at 3000 rpm (Revolutions per minute) and 4 $^{o}$C (Thermo Scientific Heraeus Primo R centrifuge). The supernatant serum was then harvested, and stored frozen in polystyrene tubes at -80 $^{o}$C for later analysis.

*b. Fasting glucose and insulin levels, HOMA-IR and QUICKI*

Blood samples were allowed to completely thaw overnight at 4 $^{o}$C, and insulin was analysed using ELISA (Enzyme-linked immuno-sorbent assay) insulin kits (Genway Biotech, Inc) at QMU lab. Reagents and standards were purchased.
Insulin ELISA is a sandwich technique based on the principle of solid phase two-site enzyme immunoassay, in which two monoclonal antibodies react against antigenic determinants of insulin. To perform this method, 25µl of insulin samples, control and standards were dispensed into microwells, and 100 µg of working insulin enzyme conjugate was added to all wells. This step was followed by mixing and incubation for 60 minutes at room temperature (18-26$^{o}$ C), to allow insulin to react with the 2 antibodies: the enzyme HRP (Horseradish peroxidase**)-**conjugated anti-insulin antibody and the anti-insulin antibody, which are both coated in the microwells. The amount of antibodies that bind to the conjugate varies depending on insulin concentration. Subsequently, wells were washed 3 times with a wash buffer (to remove the unbound enzyme labelled antibody), and 100 µl of TMB (Tetramethylbenzidine) substrate was added to all wells. Incubation was allowed

again for 15 minutes. The HRP complex was then identified by its reaction with the substrate and the development of blue colour, which intensity is inversely proportional to insulin concentration in the samples. The reaction was stopped by the addition of a stop solution (50 µl of acid) and mixing. The concentration was determined by comparing the absorbance of the samples against the standards using a microplate reader at 450 nm (Dynex technologies MRX microplate reader).

The analysis of glucose samples was undertaken at QMRI (Queen's Medical Research Institute) - Edinburgh using an automated platform (Cobas UK). Measurement of fasting blood glucose was intended for: 1) The validation that participants adhered to the inclusion criteria and were non diabetic. 2) The use of fasting blood glucose values to determine insulin resistance/sensitivity markers (HOMA-IR and QUICKI calculations). 3) The monitoring of the effect of placebo DC/PRDC on glucose levels.

Although of clinical importance, it is important to be aware of the limitations of blood glucose test, particularly because of the day-to day intraindividual fluctuations in glucose levels, which might vary between 5.7 - 8.3 % in a non diabetic person (Sacks 2011). Other limitations include acute stress or illness preceding the test (might lead to transient hyperglycemia), or the adoption of a hypocaloric diet few days before the test (might underestimate actual blood glucose levels). Analytical variations as well as the source of blood (capillary or venous) might also result in potential differences between studies. In fact, capillary blood glucose levels can be 20-25 % higher than venous blood levels (Sacks 2011). The cut-offs for fasting glucose levels are between 3.9-5.5 mmol/l. Levels higher than 5.6 mmol/l suggest a prediabetes state, whereas levels above 7 mmol/l represent a diagnosis for diabetes (Diabetes UK 2013).

The fasting plasma glucose and insulin levels were then used to assess insulin resistance/sensitivity via HOMA-IR and QUICKI, which were calculated according to the following equations:

**HOMA-IR= (Fasting Glucose (mmol/l) x Fasting Insulin (µIU/ml) / 22.5)** (Mathews et al. 1985)

**QUICKI= 1/ [log (I0) +log (G0)]**

I0 is fasting insulin (µIU/ml) and G0 is fasting glucose (mmol/l) (Katz et al. 2000).

HOMA-IR and QUICKI are the most commonly used methods for the determination of insulin sensitivity and IR. The higher the HOMA-IR index, the lower the insulin sensitivity and the higher the IR (Bonora et al. 2002). The cut-offs for HOMA-IR have not been clearly established as in the case of serum lipids and glucose levels. Studies define a normal HOMA-IR as below 3.80 (Qu et al 2011), 3.60 (Stern et al 2005), or even below 2.5 according to the American diabetes association (ADA 2010). QUICKI was shown to be an accurate index of insulin sensitivity in humans, and its cut-offs are also not clearly established. An increase in QUICKI predicts an increase in insulin sensitivity (Chen et al. 2005). It is estimated that the cut-offs for QUICKI are above 0.357 (Hydrie et al. 2012; Hřebíček et al. 2002), or 0.4 (Baldelli et al. 2007).

HOMA-IR was reported to be a more accurate index than QUICKI in assessing IR/insulin sensitivity (Keskin et al 2005; Kurtoglu et al 2010). Other studies documented that QUICKI is more reliable than HOMA-IR (Chen et al 2005; Antuna-Puente et al 2008). Yet, more studies found that these two indices are equally sensitive in assessing IR/insulin sensitivity (Cutfield et al. 2003; Menik et al 2006). Therefore, assessing both parameters might help in providing reliable results for insulin responses throughout the chocolate intervention.

The estimation of HOMA-β index as an indicator of pancreatic β-cell function might also be useful in determining any potential change in the activity of β-cells resulting from changes in insulin and/or glucose levels. HOMA- β was calculated according to the following:

HOMA-β= (20 * Fasting Insulin (μIU/mL) / (Fasting glucose (mmol/l) - 3.5)

(Matthews et al 1985)

Also called homeostatic responsivity index, HOMA-β constitutes a simple tool for assessing pancreatic β-cell function. However, the limitations of this test remain in the fact that it measures insulin secretion under non stimulated conditions. Therefore, the measurement of HOMA-β is based on the prevalent insulin action (fasting state) (Cobelli et al. 2007).

*c. Serum lipid profile (TC, HDL, LDL and TG)*

The fasting blood lipids (Total Cholesterol, HDL and TG levels) were analysed at QMRI, Edinburgh. Samples were processed using an automated platform (Cobas UK). The normal ranges for lipids in healthy individuals are presented in table 2.6.

LDL levels were then calculated via Friedewald's formula:

**LDL-C=TC-HDL-C – (TG/2.2)** (Vujovic et al. 2010)

Measurement of lipid levels was collected at a fasting state, due to the fact that TG levels remain elevated few hours following a meal (Nigam 2011). This avoided misleading evaluation of TG levels, and consequently LDL levels. Other factors that might affect lipid levels include prolonged application of tourniquet (might increase cholesterol), and infection or inflammation preceding the test (might decrease cholesterol and HDL levels) (Alvarez and Ramos 1986). This might influence the monitoring of changes in lipid levels throughout the intervention.

**Table 2.6: Normal serum lipid levels ranges**

| **Lipids levels** | **Normal ranges (mmol/l)** |
|---|---|
| Total cholesterol levels. | < 5.0 |
| LDL levels | <3.0 |
| HDL levels | >1.0 |
| Triglyceride levels | <1.7 |

(Adapted from NHS 2013). *HDL: High density lipoproteins, LDL: Low density lipoproteins*

*d. High sensitivity CRP (Hs-CRP)*

This marker was analysed at QMU lab via high sensitivity enzyme immunoassay (ELISA method) (Genway Biotech Inc). This assay uses a mouse monoclonal anti-CRP antibody and a goat anti-CRP antibody as an antibody-enzyme conjugate solution. The test consists of a reaction between the CRP molecules in the blood samples and the two antibodies during incubation. The amount of antibodies that bind to the conjugate varies depending on CRP concentration. The unbound antibodies are then removed, and the complex CRP-antibodies is allowed to react with a substrate, and the reaction is stopped by the addition of an acid.
For this assay, blood samples were allowed to completely thaw overnight at 4 $^{o}$C. Reagents were purchased. Samples were diluted 50 times with a sample diluent, and 10 µl of each sample was dispensed into the microwells (microwells were coated with mouse monoclonal anti-CRP antibody) along with 100 µl of CRP enzyme conjugate reagent, and incubated for 45 minutes. The plates were then washed five times, and TMB substrate reagent (100 µl) was added and allowed to incubate for 20 minutes, leading to the development of blue colour. The concentration of CRP in the samples is inversely proportional to the intensity of blue colour of blood samples. The reaction was then stopped by the addition of 100 µl of 1N HCl, changing the colour to yellow. The concentration was determined by comparing the absorbance of the samples against the standards using a microplate reader at 450 nm (Dynex technologies MRX microplate reader).
Hs-CRP has been shown to be a strong predictor of stroke and myocardial infarction in healthy men and women, and its importance is rising regarding primary prevention (Ridker 2001). It has been suggested that hs-CRP levels <1 mg/l represent a lower relative risk of future CVD risk, while levels between 1-3 mg/l and over 3 mg/l represent a moderate and high risk, respectively. Despite its importance in clinical settings, measurement of hs-CRP is subject to between subject and within-subject standard deviation (McCormack and Allan 2010). It has even been stated that a change in hs-CRP levels can only be effectively considered if the magnitude of increase and decrease of this marker is by at least 120% (Macy et al. 1997). Also, levels can be reliable when this measurement is taken for the same subject on 10 different blood sample occasions (Campbell et al. 2002). Furthermore, cases like

acute infections or inflammatory conditions such as rheumatoid arthritis might increase hs-CRP levels (Ridker 2001). Therefore, an elevation in hs-CRP might be non specific, and should only be interpreted along with patient history and a comparison with other hs-CRP values from the same person.

*e. LDL oxidation levels*

LDL oxidation levels were analysed at QMU lab using the Human LDL ELISA Quantification Kits (Genway Biotech, Inc) at QMU lab. Reagents were purchased. Assay optimization was first performed to ensure samples fit in the specified detection range.

For this assay, blood samples were first allowed to completely thaw overnight at 4 $^{o}$C. The process started by coating the plates with 100 µl of capture antibody (diluted in coating buffer), and incubating for 60 minutes. This was followed by washing the plates (3 times) and adding 200 µl of block buffer to all wells. Afterwards, solutions of protein calibrator (containing human LDL antigen) and diluted samples (samples were diluted 30 times) were assigned into the wells, and incubated for 60 minutes. Following the second incubation, wells were washed 5 times, and 100 µl of detection antibody (HRP-conjugated anti-LDL antibody) was added to all wells. After washing the plates again for 5 times, 100 µl of TMB solution (substrate) was added to all wells. The plates were incubated again for 20 minutes, to allow the development of blue colour which intensity is inversely proportional to LDL concentration in the sample. The reaction was stopped by the addition of 100 µl of stop acid solution. The concentration was determined by comparing the absorbance of the samples against the standards via a microplate reader at 450 nm (Dynex technologies MRX microplate reader).

Regarding normal serum oxidised LDL levels, results are still conflicting. A study showed that in healthy individuals, the mean value of oxidised LDL was 95.32 ± 37.85 ng of ox-LDL/ml (Koubaa et al 2007). Another study showed that the mean oxidised LDL levels in 120 healthy individuals were 78 ± 23.7 ng/ml, while these levels were 139.6 ± 52.2 ng/ml in diabetics (Koubaa et al 2011). It won't be entirely reliable to assess cardiovascular risk based on the values of oxidised LDL, but this

test would most likely be a valid tool to monitor the effect of an intervention over time.

#### 2.3.5.5 Saliva tests

For the analysis of cortisol and cortisone levels, participants were asked to provide 2 saliva samples on each appointment: The first sample upon waking and the second sample at noon. Participants were instructed to provide sufficient amounts of saliva by spitting into a collection tube, and to avoid being stressed the time they gave the samples. They were also asked to avoid drinking alcohol during the collection day. Samples were then stored frozen at -18$^{o}$C for later analysis.

The analysis of steroids from saliva has been an increasingly used method, as it is simple and non invasive (Groschi 2008). It also presents advantages over the analysis of steroids in the urine due to the burden associated with urine collection (Yehuda et al. 2003). Studies showed that a saliva sample at wake-up provides nearly a good estimate of the 24-hour urinary cortisol excretion, as well as the overall daily cortisol production (Neary et al. 2002; Yehuda et al. 2003). Another study showed that the reliability of saliva in measuring cortisol levels is higher when several saliva samples are collected at different intervals during the day (Groschi 2008). These studies suggest that saliva may be alternative to urine in analysing glucocorticoid hormones (Yehuda et al. 2003), and subsequently the activity of 11-β HSD1.

The activity of the enzyme 11β-HSD1 was determined by the ratio cortisol/cortisone. An increase in this ratio suggests an increased expression of this enzyme, and consequently an increased regeneration of cortisol from cortisone in the liver and adipose tissue (Sigurjonsdottir 2009).

*a. Extraction of saliva samples*

Saliva samples were first thawed and centrifuged at 3000 rpm for 10 minutes to get rid of any impurities. Afterwards, an aliquot of 250 µl of saliva was pipetted into

Fisher extraction tubes. Diethylether was then added (up to 1.5 ml), and the samples were vortexed by a multi-tube vortexer for 10 minutes, and stored at -80$^{o}$C for 6 minutes. Freezing allowed the separation of the aqueous fraction from the ether-steroid fraction, the latter one being decanted into Pyrex glass tubes. The ether was then evaporated using nitrogen. After evaporation, the samples were reconstituted with 250 µl of assay buffer (concentration equal to the saliva sample), vortexed again by a multi-tube vortexer, and left in the fridge overnight at 4 $^{o}$C. The samples were then ready for the ELISA assay.

*b. Cortisol and cortisone levels: ELISA assay*

*b.1. Principle of ELISA assay*

Cortisol and cortisone levels were analysed through the "in-house ELISA method" a method developed by Al-Dujaili (2006). The method is an indirect competitive double-sandwich ELISA between the conjugate (antigen) and cortisol/cortisone in the samples for the antibodies binding sites. The amount of antibodies that bind to the conjugate varies depending on the cortisol/cortisone concentrations. The bound hormones are detected by the addition of a substrate and the production of a blue colour. The colour intensity is inversely proportional to cortisol/cortisone concentrations in the samples. Stopping the reaction by the addition of an acid allows the determination of the concentration of glucocorticoids by comparing the absorbance of the samples against the standards via a microplate reader.

*b.2. Preparation of reagents*

For this method, coating buffer, wash buffer, block buffer, assay buffer, enzymes, antibodies and standards were prepared in the Lab, whereas the substrate was purchased.

Coating buffer

Coating buffer was prepared by adding PBS (phosphate buffer saline) to distilled water (1 : 1.5 dilution). The buffer was stored at $4^{o}$C for later analysis.

Wash buffer

Wash buffer was prepared by adding 0.5 ml of Tween 20 solution (soap) to 800 ml of distilled water and 200 ml of PBS solution (1:5 dilution).

Block buffer

Block buffer was prepared by adding 0.5 g of BSA (bovine serum albumin) to 100 ml of PBS (0.5 % concentration).

Assay buffer

Assay buffer was prepared by diluting block buffer five times (1:5 solution)

Preparation of antibodies

The preparation of antibody solutions was made by adding 11 ml of assay buffer to 50 µl of cortisol/ cortisone antibodies (1/ 20000 solution).

Preparation of enzymes

Enzymes were prepared by adding 11 ml of assay buffer to 10 µl of anti-sheep enzyme (1:10000 for cortisol), and to 20 µl of anti-rabbit enzyme (1:5000 cortisone).

Preparation of standards

The preparation of standards was made by performing serial dilutions of the cortisol and cortisone stocks in assay buffer. For cortisol, the following concentrations were generated: 0, 2.5, 10, 50, 250 and 1000 ng/ml. For cortisone, the concentrations of the standards were 0, 0.5, 1.0, 2.5 and 10 and 50 ng/ml.

*b.3. ELISA assay*

First, 96-well microplates were coated with cortisol/cortisone conjugates. This was done by diluting 50 µl (0.5 mg/ml) of cortisol-conjugated to BSA or 50µl (0.5mg/ml) of cortisone-conjugated to 20 ml of coating buffer, and adding 200 µl of diluted cortisol-conjugated to BSA or 180 µl of cortisone-conjugated solution to the wells (Al Dujaili et al. 2012). The plate was allowed to incubate overnight at $4^{o}$C. The next day, fluid was discarded from the microplates, which were washed 4 times with 250 µl of wash buffer. Then, 200 µl of bloc buffer was added to the microplates, the latter were then covered and incubated for 1 hour at 37 $^{o}$C. The fluid was subsequently discarded, and 50µl of saliva samples were added to the plates, along with 100 µl of antibody (anti-sheep for cortisol and anti-rabbit for cortisone). The plates were covered and allowed to incubate in a dark place for 2 hours at room temperature. Following incubation, the fluid was discarded again, and the plates washed 4 times with 250 µl of wash buffer. Then, 100 µl of enzyme was added (anti-sheep for cortisol and anti-rabbit for cortisone) to the microplates, which were allowed to incubate again for one hour at room temperature and in a dark place. Microplates were then washed again four times, and 100 µl of substrate solution was added, followed by 15 minutes incubation. During this time, a light blue colour was developed, and the reaction was stopped by adding 50 µl of stop solution (2 M $H_2SO_4$). The absorbance was read at 450 nm using a microplate reader (Dynex technologies MRX microplate reader).

### 2.3.6 Compliance and diet diaries collection

In order to assess changes in dietary intake following DC consumption, participants were asked to provide a 3-day diet diary (two weekdays and one weekend) during the run-in period and at week 3 (Appendix 5). Information on how to fill the diet diaries were provided by the researcher. Diet diaries were then analysed using the Netwisp Software V3.0 (Tinuviel software) for total energy, carbohydrate, fat and protein.

Diet diaries represent a simple and easy method to collect food intake, and it does not depend on memory. The multiple-day diet diary provides a better estimate of the

habitual intake of participants, compared to 24-hour recall or 1-day diet diary. However, this method is limited by the non compliance due to the time and effort needed to record food intake, and the possible change in dietary intake during the recording to decrease burden and complexity (Lee and Nieman 2010). Also, the flat slope effect (underestimation of large portion sizes and overestimation of small portion sizes) (Garriguet 2008) might produce bias in actual food intake. This might threaten the efficacy of this method in assessing diet (Bothwell et al. 2009).
The validation of diet diaries has been previously made via several methods such as doubly-labelled water, estimation of nitrogen excretion and estimation of energy expenditure. Studies have mainly showN an underestimation of actual energy intake and an underreporting in the general population by a mean of 22 % (Garriguet 2008). Many factors have been associated with underreporting, such as age, sex and socioeconomic and psychological factors. In fact, women compared to men, and physically active individuals have been documented to have a higher level of underreporting. However, the highest degree of underreporting was observed in obese individuals, whom reported energy intake constituted 59 ± 24 % of their energy expenditure (Garriguet 2008). These factors might affect the validity of diet diaries in assessing energy intake.
Nonetheless, since there is no best method for analysing food intake (Lee and Nieman 2012), diet diaries are still widely used in research studies to estimate changes in dietary intake throughout interventions (Wrieden et al. 2003).

Compliance to the study protocol was assessed: 1) through the 3-day diet diary and physical activity questionnaire 2) by the return of the empty chocolate containers at the end of the intervention and 3) by directly asking participants whether they have consumed all the samples of chocolate daily as required. As far as we know, there is no previous description of a high compliance to a DC intervention. Therefore, a high compliance was defined in this study by the intake of 85 % or more (equivalent to missing no more than one sample a week) of the chocolate throughout the study.

### 2.3.7 Statistical analysis

Continuous normally distributed data were expressed as mean ± SD, unless otherwise stated (95% confidence intervals and interquartile range (IQR) when appropriate). Data were analysed using SPSS for Windows version 19.0 (SPSS, Chicago, IL). For within group comparisons, changes from baseline were analysed using a student's paired t-test. Differences in baseline characteristics between groups were examined using a two-tailed samples independent t-test. For between-group differences, analysis of covariance (ANCOVA) was carried out to adjust for potential baseline differences. Paired t-tests and independent t-tests on the Netwisp software (V 3.0) were used to assess within group differences and baseline differences, respectively between the 2 groups with regards to energy, protein, fat and carbohydrates. The relationship between selected variables was examined using Pearson's product-moment correlation coefficient (r). Non parametric data were analysed using Wilcoxon and Mann Whitney tests. Significant changes were set at $p \leq 0.05$.

## 2.4 Study II

### 2.4.1 Study population

Advertisement for the study was made via QMU university moderator as well as community centres in East Lothian, hospitals in Edinburgh, gyms in Musselburgh and word-of mouth.

**Inclusion and exclusion criteria**

Fourteen volunteers without restriction to race or socioeconomic status were recruited according to the following criteria:

***Inclusion criteria***

- Overweight with no history of diabetes, hypertension or CVD
- BMI between 25 – 34.9 $Kg/m^2$
- Males and Females

- Age: 18-65 years

***Exclusion criteria***

-Participants with cardiovascular diseases, hypertension or diabetes

-Participants taking medications that affect insulin, glucose, lipid or blood pressure levels

-Participants taking dietary supplements containing high doses of antioxidants

-Postmenopausal women taking HRT (Hormone Replacement Therapy)

-Participants who recently participated or are currently on a weight management program

-Smokers and heavy alcohol drinkers (defined as more than 15 drinks/week for men and more than 8 drinks/week for women (CDC 2014))

-Participants with regular consumption of cocoa or DC (> 1 serving/week) (Taubert et al. 2007).

### 2.4.2 Determination of sample size for the small scale study

In order to get an approximate number of participants for this study, the determination of sample size was based on its ability to have 80% power to detect an effect size of 1.325 using a two group t-test with a 0.050 two-sided significance level. Hence, for a two group trial, the total sample size required was 20, and with 30% attrition (based on a weight loss study by Drummond et al. 2004), 28 participants were supposed to be recruited in total.

### 2.4.3 Estimation of sample size for a larger study based on Study II results

One of the aims of this study was to estimate a sample size for a larger scale study. Delucchi (2004) discussed the importance of appropriately estimating sample size to perform a meaningful study. Commonly, the determination of the number of participants necessary to detect an effect is done via conducting a power analysis, given the availability of an effect size from previous studies (IDRE 2014). Power analysis has also few disadvantages, like the lack of generalizability of this tool, as it depends on the type of statistical analysis used. When running a power analysis, it is

recommended to use different values of means and standard deviations in order to obtain a range of numbers of sample sizes (IDRE 2014). Many references for sample size calculations were suggested in the literature (examples Muller et al. 1992; Machin et al. 1997; Rochon 1998).

### 2.4.4 Study design

This study is a randomized single-blinded placebo controlled pre-post intervention parallel study with 2 arms where participants received two different types of DC according to the following:

Type A: Placebo DC (low in polyphenols)
Type B: DC rich in polyphenols (500 mg)

Randomization was implemented using sealed envelopes based on the protocol described by Doig and Simpson (2005). Twenty-eight standard-sized sheets were marked by either P (Placebo)-14 sheets or D (Dark chocolate rich in polyphenols)-14 sheets. The sheets were inserted into unlabelled envelopes and sealed and placed into a container. Then, the researcher chose an envelope for each participant.
Participants were provided an information sheet and asked to sign a consent form (Appendix 7) before the start of the study. The study took place at QMU clinical lab. Each of the 3 appointments and the follow-up appointment lasted no more than 20 minutes. Participants randomly received 20g of DC daily (Type A or B) for a period of 12 weeks. Anthropometric measurements (height, weight, WC, and body composition) were taken during each visit, and a 24-hour urine sample collection was required the day before each of the first 3 appointments (View table 2.7 for the timeline of each appointment). A follow-up appointment four weeks after the end of intervention was planned to identify whether any changes in anthropometric measures were maintained.
A general questionnaire (Appendix 8) was filled by participants during the run-in phase. To assess flavonoid intake, a food frequency questionnaire (FFQ) for assessment of flavonoid intake was filled four times throughout the intervention (Appendix 9). Volunteers were asked to maintain their usual diet and physical

activity one week before the start and throughout the study period. This was monitored through reporting diet diaries (at baseline and every four weeks) (Appendix 5), and filling a physical activity questionnaire (before the start and every 6 weeks) (Appendix 10). A diet adjustment was considered so that DC substitutes another food of similar energy. The researcher provided dietary advice for participants to help them achieve this goal. This happened by asking participants about the types of snacks usually consumed. Energy composition of these snacks was assessed, and substitutions based on food energy content were suggested by the researcher. A short questionnaire on the acceptability of the treatment and the potential change in appetite was filled during the 3rd appointment (Appendix 11) (View Figure 2.5 for the study protocol). Volunteers were requested to refrain from consuming any other type of dark chocolate or cocoa for the whole period of the study. Females were asked about the date of their menstrual cycle on each appointment. The details of all data collected are discussed in section 3.4.6.

Before attending the appointments, participants were asked to:

-Be on a fasting state (defined as to refrain from eating or drinking any liquids for a period of 8 hours (Diabetes UK 2013).
-Avoid taking high quantities of caffeine from caffeine rich sources (mainly coffee and tea) 12 hours before the appointment
-Avoid drinking alcohol for the past 24 hours.

**Table 2.7: Timeline for Study II appointment**

| | Minutes |
|---|---|
| Introduction | 5 |
| Height and weight measurements | 3 |
| Waist circumference measurement | 4 |
| Bioelectrical impedance | 6 |
| Questions | 2 |
| Total | 20 |

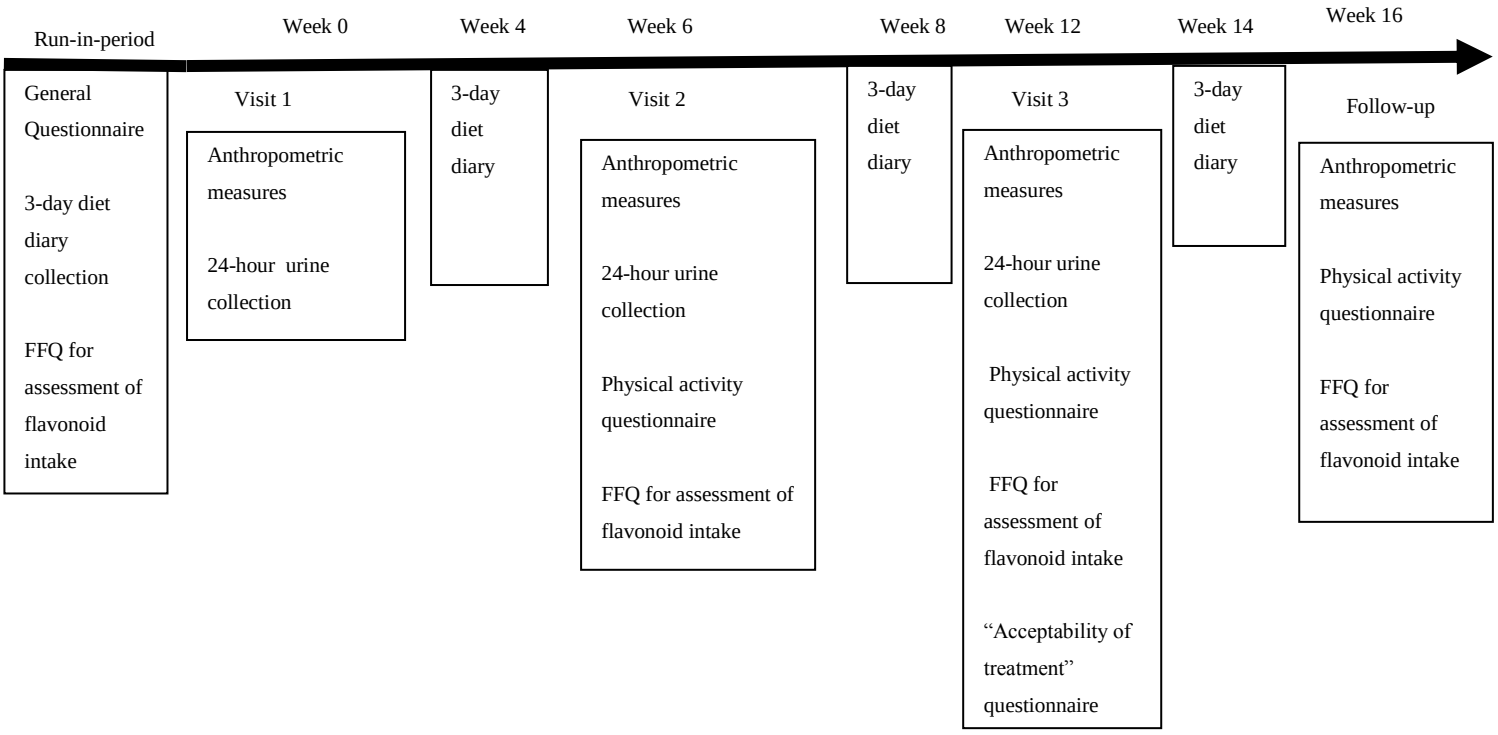



**Figure 2.5: Study II protocol**

*FFQ: Food Frequency questionnaire.*
*Anthropometric measures included weight, height, waist circumference and body composition.*

### 2.4.5 Justification for the chosen methods

#### 2.4.5.1 DC dose and study duration

No previous human studies have discussed the dose of DC and polyphenols supposed to induce an effect on body weight. There has been one study performed on 26 overweight and obese premenopausal women, who were administered either a dark chocolate snack or non-chocolate snack daily for 18 weeks. This study showed that a sweet daily snack, whether it is dark chocolate or non chocolate, helps in decreasing body weight, body fat, and waist and hip circumference in overweight and obese women (Piehowski et al. 2011). Yet, it was not possible to rely on it to determine the duration of the study, as no assessment of the dependant variables (BMI, WC and body composition) was undertaken at different intervals of the study, hence it was not clear whether changes were significant before week 18, e.g. at week

6 or 12. Moreover, the polyphenol content of the chocolate was not quantified, and the design of the study is different as it addresses the effect of daily sweet snack consumption versus dark chocolate consumption on anthropometric measures, when these 2 snacks were incorporated into a reduced calorie diet.
In order to determine the study duration, data on previous studies performed on green tea (rich in polyphenols) and its effect of body weight were used. Three studies aiming to determine the effect of daily green tea supplementation (containing about 500-600 mg of polyphenols) on anthropometric measures were shown to lower body weight, waist circumference and body fat mass after 12 weeks of daily supplementation (Grove et al. 2010). Knowing that green tea does not provide energy, the purpose of the study was to substitute DC by another food in order to maintain the daily energy intake of participants. Also, it is known that polyphenols in cocoa/DC have a different composition than green tea. In addition to its -(-)epicatechin content, tea contains 30 - 50 % of EGCG (Grove et al. 2010). Hence, information from these studies was mainly used to get an approximate idea of the study duration.

Regarding the dose of DC and polyphenols, an animal study noted that a weight-lowering effect could be possibly achieved in humans by consuming 50g of cocoa daily (provides 182 Kcal; the amount of polyphenols was not specified)) (Matsui et al. 2005). Another animal study stated that this amount is equivalent to 54 g of cocoa powder (provides 221.4 Kcal and contains 513 mg of polyphenols and 24.84 mg of epicatechin) (Gu et al. 2013).
In view of these data, the choice of a 20g daily dose of chocolate containing 500 mg of polyphenols with 85 mg of epicatechin and providing 102 Kcal was tested for its ability to induce a decrease in weight in humans over a period of 12 weeks.

#### 2.4.5.2 Study design

The choice of a parallel design was primarily based on the long study duration (12 weeks) (View section 3.3.4.2 on the advantages of parallel studies compared to crossover studies). This made the choice of a crossover design impractical due to the high commitment asked from participants. In addition, the washout period expected

to eliminate carry over effects between the two treatments could range between 2 and 6 weeks in studies lasting 8-12 weeks (Ried et al. 2009; Sathyayapalan et al. 2010; De Bock 2013). Therefore, the choice of a parallel study was deemed to be beneficial in avoiding an increase in dropouts and a decrease in compliance (Dash et al. 2012).

### 2.4.6 Data collection and processing

#### 2.4.6.1 General questionnaire and Food frequency questionnaire

The general questionnaire was described in section 2.3.5.1 and presented in appendix 8. Similarly to Study 1, the questionnaire covered information on social characteristics, lifestyle habits and medical history, and was designed to ensure participants met the study requirements.

Participants noted their actual intake of flavonoid-rich foods in a FFQ with 43 food items adapted from USDA database of the flavonoid content of selected foods (2011a) (Appendix 9). Foods with a medium and high content of flavonoids (more than 4 mg/100g of foods) were included (a medium content of flavonoids in foods is between 4 - 9.9 mg/100g and a high content constitute more than 10mg/100g of selected foods (O'byrne et al. 2002)). A portion size based on the average serving for each food was stated, and participants were asked to tick one of the nine options ranging from "Never or less than once a month" to "more than 6 per day". Each food item was assigned a score based on the average intake in the following way:

Never or less than once a month: **0**
1-3 per month: **2**
Once a week: **4.28**
2-4 per week: **12.86**
5-6 per week: **23.57**
Once a day: **30**
2-3 per day: **75**
4-5 per day: **135**

More than 6 per day: **180**

A score was also assigned to whether the food was medium or high in flavonoids, so that foods containing 4- 9.9 /100 g of foods were given a score of 1, and foods with content higher than 10 mg/100g of foods were given a score of 2. Participants were asked to fill the FFQ four times throughout the intervention. An increase in flavonoid intake was positively correlated to an increase in FFQ score.

FFQ is considered a simple and easy way to determine dietary intake (Santos-Buelga 2010). Many studies have previously discussed the validity and reproducibility of FFQ in assessing polyphenol intake (Hakim et al. 2001; Rautiainen et al. 2008; Vian et al. 2013). A significant association between FFQ for assessment of polyphenol intake and urinary polyphenol excretion (pearson's (r) = 0.23; $p = 0.01$) (Vian et al. 2013), as well as between FFQ for assessment of polyphenol intake and FRAP levels (pearson's (r), $p<0.05$) (Rautiainen et al. 2008) were previously noted. FFQ was also validated for assessing flavonoid intake (Otaki et al. 2009). Nonetheless, the ability of FFQ to assess dietary intake was criticized. This was mainly explained by the bias in self-reporting related to the quantification of portions by participants. Commonly, participants tend to overestimate their intake of healthy foods like fruits and vegetables (Spencer et al. 2008), which can lead to an overestimation of the intake of polyphenols. This makes it difficult to assess the correlation between diet and diseases (Byers 2001). Furthermore, the big variety of products in the market as well as the different ingredients and cooking techniques produce errors in the ability of FFQ to accurately assess polyphenol intake. Yet, despite its limitations, FFQ is still considered a valid and useful tool to determine changes in flavonoid intake (Santo-Buelga et al. 2010)

It is important to mention that FFQ used in this study did not aim to analyze the total flavonoid intake in this population, but to compare the intra individual changes in the intake of flavonoids throughout the study. Therefore, low flavonoid foods (< 4 mg/100 g of selected foods) were not included.

#### 2.4.6.2 Physical activity and acceptability questionnaires

The questionnaires were described in section 3.3.5.1. Physical activity questionnaires were filled four times during the intervention (Appendix 10). Baseline physical activity questions were included in the general questionnaire. Hours of physical activity were then transformed into MET/week. Data regarding acceptability of dark chocolate consumption and the willingness to continue having chocolate after the study were obtained. A question about potential changes in appetite throughout the study was also included (Appendix 11).

#### 2.4.6.3 Anthropometry

*a. Height, weight and waist circumference*

The description of these methods is presented in section 3.3.5.2.

*b. Body composition*

Body fat percentage and LBM were measured via bioelectrical impedance (BIA) using the BODYSTAT 1500 MDD (BODYSTAT®). This machine also provided a measure of total body water (TBW).
Measurements were taken in a warm room, with participants lying in the supine position. Sets of electrodes were placed on the right hand and right foot, and measurements were taken with no parts of the body touching one another.
BIA measures impedance (defined as an opposition to a flow of a current) by involving a small electric current passing through the body at a speed that is dependent on body composition. Water and ions are good conductors of the electricity, thus muscles and bones are more conductive than adipose tissue. The higher the lean body mass, the higher the conductivity and the lesser the impedance. On the other hand, fat mass is inversely correlated with conductivity, and directly correlated with impedance (Dehghan and Merchant 2008).

BIA was reported to be a reliable instrument for the assessment of body composition as it is quick, simple and non invasive (Walter-Kroker et al. 2011). BIA presents less intra and inter-observer error compared to other techniques such as skinfold

thickness and waist circumference, and it has been validated against reference methods in the Caucasian population (Lopez et al. 2012). It was estimated that BIA is highly reproducible with less than 1% error (Segal et al. 1991). BIA can be confounded by factors such as exercise (less than 2-3 hours before measurement) and food consumption (less than 3 hours prior to the test). In addition, BIA technique may be affected by the hydration status of individuals (can affect the estimation of fat free mass) (Lukaski et al. 1986), environmental factors (like skin temperature which implies an inverse correlation between skin temperature and impedance (Gudivaka et al. 1996)), and the menstrual cycle in women (which may result in a higher within-subject difference compared to men, due a change in the hydration status throughout the cycle (Dehghan and Merchant 2008). Desirable ranges of fat are presented in table 2.8.

**Table 2.8: Desirable ranges of body fat percentage in adult males and females**

| Classification | Males | Females |
|---|---|---|
| Unhealthy (too low) | ≤ 5% | ≤ 8% |
| Acceptable (lower end) | 6-15% | 9-23% |
| Acceptable (upper end) | 16-24% | 24-31% |
| Unhealthy (too high) | ≥25% | ≥32% |

*Adapted from Lee and Nieman (2010). Ranges are for individuals aged 18 years and older and based on gender.*

*c. Basic metabolic rate (BMR)*

BMR was estimated via the BIA machine after an overnight fast and 12 hour abstinence from exercise. As the accurate measurement of BMR requires methods such as indirect calorimetry which measures oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) (McClave and Snider 1992), the values obtained via BIA machine provide estimations based on the formula of Brozek and Grande (1955). This formula uses lean body mass to calculate BMR rather than total body weight. BMR is affected by several factors such as caffeine intake, exercise, and anxiety. Repeated measures of this test on two days and preferably in the morning, are likely

to provide more accurate results (Haugen et al. 2003). Studies have previously documented the practicality of BIA in estimating changes in BMR levels in routine measurements (Barak et al. 2003; Altay et al. 2012), making it a useful tool for assessing changes in energy expenditure during this study.

#### 2.4.6.4 Urine tests

##### *a. Processing of urine samples*

Urine samples were first collected and frozen at -18$^{o}$C. Prior to analysis, samples were thawed at 4$^{o}$C and centrifuged at 6000 rpm to get rid of any impurities, and used for conducting the FRAP, Folin-Ciocalteu and ELISA methods.

##### *b. Cortisol and cortisone extraction*

Before conducting the ELISA method, cortisol and cortisone were extracted based on the technique described by Al Dujaili (2006). 1ml of urine was added to 8 ml of dichloromethane, and the mixture was vortexed for 10 minutes using a V400 multi-tube Vortexer. Subsequently, sodium hydroxide (NaOH) was added to the mixture and the samples were inverted 3 times. NaOH was then discarded and hydrochloric acid (HCl) was added and discarded again. This procedure was repeated while replacing NaOH and HCl with water. The resultant sample containing a high amount of steroids was transferred to Pyrex glass tubes, and dichloromethane was evaporated using Nitrogen. Samples were then reconstituted with 1 ml of assay buffer and left overnight at 4$^{o}$C.
The principle of ELISA method for the determination of cortisol and cortisone levels is described in section 3.3.5.5.

The use of urine samples for the determination of glucocorticoids levels is mainly due to the fact that it is a non invasive technique (Yehuda et al. 2003), and is highly correlated to blood cortisol (Neary et al. 2002). In fact, a 24-hour sample provides a good estimate of the amount of cortisol secreted by the adrenals over a complete circadian cycle. However, some limitations are inherent to this method. Some unidentified substances might interfere with the extraction of cortisol, and lead to

falsely high cortisol values. Also, the burden of collecting urine may result in a decrease in adherence, and might consequently affect the reliability of this test (Yehuda et al. 2003).

*c. Total antioxidant capacity: Ferric-reducing antioxidant power assay*

Ferric-reducing antioxidant power (FRAP) assay is a method used to assess the antioxidant capacity in foods and biological samples. The principle is based on the analysis of reduced agents based on electron transfer (e) according to the following reaction:

Probe (oxidant) + e (from antioxidant) $\longrightarrow$ Reduced probe + oxidised antioxidant

In this reaction, the probe is an oxidant (ferric-2,4,6-tri-2-pyridyl-s-triazine complex ($Fe^{3+}$ TPTZ) that takes off an electron from the antioxidant, causing its change in colour to blue. Therefore, the measurement of the antioxidant capacity of phenols against the oxidant will measure the ability of the solution to reduce $Fe^{3+}$ TPTZ to $Fe^{2+}$ TPTZ. The magnitude of change of colour is proportional to the antioxidant concentration, and the reaction reaches its endpoint when the change in colour stops. FRAP assay is carried out under acidic solutions to maintain iron solubility.

c.1. Preparation of reagents

For this technique, reagents including ferrous sulphate standards, acetate buffer, hydrochloric acid, ferrous sulphate, TPTZ, and ferric chloride solutions were prepared in the Lab.

**Ferrous sulphate standards:** To prepare the standard test tubes, 0.278g of ferrous sulphate was diluted in 1 L of deionized water (1 mM). Then, serial dilutions were performed to produce the standards showed in Table 2.9. These standards were used to produce the standard curve.

**Table 2.9: Ferrous sulphate standards**

| Standard concentration (mM) | Ferrous sulphate solution (ml) | Distilled water (ml) |
|---|---|---|
| 0.1 | 1.0 | 9.0 |
| 0.2 | 2.0 | 8.0 |
| 0.4 | 4.0 | 6.0 |
| 0.6 | 6.0 | 4.0 |
| 0.8 | 8.0 | 2.0 |
| 1.0 | 10.0 | 0.0 |

**Acetate Buffer** (300 mM, pH 3.6) was prepared by the dissolution of 3.1 g of sodium acetate and 16 ml of glacial acetic acid into 1 L of deionized water.

**Hydrochloric Acid (**40 mM) was prepared by adding 3.64 ml of concentrated hydrochloric acid (36%, 12 M) to 1 L of deionized water.

**Ferrous sulphate** (1mM) was prepared by adding 0.278 g of ferrous sulphate to 1 L of deionized water.

**TPTZ (**Ferric-2, 4, 6-tri-2-pyridyl-s-triazine) (10 mM) solution was prepared by adding 0.031g of TPTZ in 10 ml of 40 mM HCl

**Ferric Chloride** (20 mM) was prepared by dissolving 0.054 g of ferric chloride into 10 ml of deionized water.

**Working FRAP solution** was made by adding the following into a 250 ml glass bottle: 100 ml acetate buffer, 10 ml Ferric chloride, 12 ml distilled water and 10 ml TPTZ.

c.2. FRAP assay

The FRAP technique consists of adding 10µl of standards and urine samples (diluted 10 times) into a 96-well plate, followed by the addition of 250 µl of working FRAP solution to all wells. The plate was incubated in the oven (set to 37$^{o}$C) for 4 minutes, and read on the micro plate reader (Dynex technologies MRX microplate reader) at 600 nm. The change of absorbance was due to the combination of the reducing capacity of all the reacting antioxidants in the sample. The mean concentration of $Fe^{2+}$ produced was expressed in mmol/l (and then converted into mmol/day) and calculated using the following equation:

$$\mathbf{[FRAP] = p\,[Abs\ sample - b] / m}$$

**P** = dilution factor of original sample

**Abs sample** = absorption at 600 nm

**b** = intercept from standard curve

**m** = slope from standard curve

The FRAP method represents a recognized index of antioxidant power, and its advantages remain in the high reproducibility of results and the fact that it is speedy, simple and inexpensive (Benzie and Strain 1996). However, many limitations are inherent to this method. First, the FRAP technique is based on the fact that all redox reactions are complete within 4-6 minutes, albeit some polyphenols need a longer reaction time to be detected (around 30 minutes). Also, the FRAP assay is only capable of measuring the reducing capacity of the solution based on the ferric ion, hence it lacks specificity and is unable to measure thiol antioxidants (Prior et al. 2005). In addition, phosphates and sulfates, largely available in biological fluids, can bind to $Fe^{3+}$ and reduce its reduction to $Fe^{2+}$ (Cornelli et al. 2011), resulting in a possible overerestimation of the amount of antioxidants in the urine sample.

*d. Total polyphenols in the urine: Folin-Ciocalteu method*

Folin-Ciocalteu method is used to determine total polyphenols in the urine. Phenols, in addition to other oxidised substances, are determined by producing a blue colour due to the reaction with yellow heteropoly-phosphomolybdate-tungstate anions (in

the Folin-Ciocalteau reagent). To illustrate, under basic conditions (pH=10), phenols are dissociated to give phenolate anions, which can reduce the Folin-Ciocalteau reagent through an electron transfer mechanism, leading to the development of blue colour.

d.1. Preparation of reagents

For this method, gallic acid standards, sodium carbonate and Folin and Ciocalteau reagent solutions were prepared in the lab.

**Gallic Acid standards**

Gallic acid (500 mg) was added to 1 L of distilled water to produce the stock. Then, serial dilutions were performed to produce the standards shown in table 2.10. These standards were used to produce the standard curve.

**Table 2.10: Gallic acid standards**

| Gallic acid (mg) | Gallic acid solution (ml) | Distilled water (ml) |
|---|---|---|
| 500 | 10 | 0 |
| 400 | 8 | 2 |
| 300 | 6 | 4 |
| 200 | 4 | 6 |
| 100 | 2 | 8 |
| 50 | 1 | 9 |

**Sodium Carbonate** solution was prepared by adding 115 g $Na_2CO_3$ into 1 L of deionized water.

The **Folin and Ciocalteau** reagent was prepared by adding 100 ml of Folin's reagent to 900 ml distilled water (1:10 dilution).

*d.2. Folin and Ciocalteau assay*

For this assay, 100 µl of each urine sample (diluted 10 times), 5 ml of Folin's reagent, and 0.9 ml of distilled water were added to test tubes and left for 5 min. Then, 3.5 ml of $Na_2CO_3$ was added and left to develop for 2 h. This step was followed by measuring the absorbance for each sample at 765 nm against a water blank on a spectrophotometer. The concentration of total phenolics was calculated by comparing the optical density of samples against the standard curve prepared with gallic acid. Results were expressed in mg/l of GAE, and then converted into mg of GAE/day.

The Folin-Ciocalteu method has been previously used as an indicator of polyphenol content, and represents many advantages related to its convenience, reproducibility and simplicity. The limitations of this method remain in its non specificity to phenolic compounds, as other components such as vitamin C and copper can reduce the Folin-Ciocalteu reagent, and therefore overestimate the phenolic content the sample (Berker et al. 2010).

In view of the potential limitations of the FRAP and Folin-Ciocalteu techniques in assessing changes in polyphenols in the urine, the use of both methods might give an estimation of any potential change in antioxidant capacity and in the amount of polyphenols resulting from PRDC consumption.

### 2.4.7 Compliance and diet diaries collection

Compliance was assessed by 1) The measure of total polyphenols and antioxidant capacity in a 24-hour urine sample before, during and at the end of the intervention. 2) Counting the empty plastic bags and asking participants whether they have consumed all the doses of chocolate as required. 3) Monitoring fluctuations in the participants' diet and physical activity through the collection of diet diaries (Appendix 4) and physical activity questionnaires (Appendix 10). A high compliance was defined by the intake of 85 % or more (equivalent to missing no more than one

sample a week) of the experimental chocolate throughout the intervention (section 3.3.6 provides additional information about compliance and diet diaries)

### 2.4.8 Statistical analysis

Continuous normally distributed data are expressed as mean ± SD. Data were analysed using SPSS for Windows version 19.0 (SPSS, Chicago, IL). For multiple comparisons, data were analysed using mixed model ANOVA (Analysis of variance) with time (week 0, week 6, week 12 and then week 0, week 6, week 12 and week 16) as within subject factor, and treatment (placebo DC/PRDC) as between subject factor. For significant differences, changes over time were assessed via pairwise comparisons using Bonferroni test. Energy, protein, fat and carbohydrate intakes were analyzed using repeated measures ANOVA. The correlation between selected variables was examined through Pearson's product-moment correlation coefficients (r). Significance was set at $p \leq 0.05$. Sample size calculation for a large trial was calculated based on 80% power and a two-sided significance level of 0.05.

# CHAPTER 3: INTRA-OBSERVER RELIABILITY OF WAIST CIRCUMFERENCE MEASUREMENT

Before exposing the results of the conducted studies, this chapter will discuss the intra-individual error of measurement study that was carried out in order to assess the reliability of WC measurement.

Anthropometry is widely used in epidemiological and clinical studies as a simple tool to assess adiposity and weight status due to the fact that it is quick, non invasive and inexpensive (Ulijaszek and Kerr 1999). Nevertheless, due to the inadequacies in instruments, the inabilities in recording the measures (Arroyo et al. 2010), the lack of trained anthropometrists and the high intra and inter-error variability (Geeta et al. 2009), anthropometric measurements represent a high degree of error that affects precision and reliability (Sicotte et al. 2010). This error is particularly high when compared to other techniques such as haematological methods (Geeta et al. 2009). Error of measurement is defined as the degree of difference between repeated measures (Verweij et al. 2012).

Despite the importance of anthropometric measurements in clinical and community settings, there have been few reliability studies assessing errors of these measurements in the adult population (Geeta et al. 2009; Arroyo et al. 2010). Estimating errors in anthropometry is particularly important to improve measurements and properly estimate the correlation between variables in clinical trials (Bland and Altman 1986).

WC has been increasingly used to monitor the effect of an intervention on anthropometric measures (Verweij et al. 2012). However, as mentioned in section 1.1, because of the lack of a uniform protocol for the measurement of WC (Mason and Katzmarzyk 2009) (fourteen protocols have been identified (Geeta et al. 2009)), BMI is still the most widely used tool for assessing weight status. As simple as it seems, measurement of WC by clinicians has been reported to be difficult (Mason and Katzmarzyk 2009). Training anthropometrists, repeating measurements (two

times and preferably 3 times), and establishing a uniform protocol are necessary to reduce the error (Verweij et al. 2012).

Studies assessing measurement error in WC are limited. Therefore, it has been suggested that researchers carry out their own reliability studies to ensure accuracy (Pelletier et al. 1991), reliability and precision of WC measurement.

Intra observer precision is defined as the degree of difference between measures carried out on the same person by the same observer, whereas inter observer precision is the extent of difference when the measurement is conducted by two or more observers (Ulijaszek and Kerr 1999). In addition, the terms validity and reliability have been used to define error of measurements in anthropometric measures. Validity refers to the degree of which the true measurement is achieved, while reliability is defined as the ability of differentiation between participants of the same study regardless of the error of measurement (Geeta et al. 2009; Verweij et al. 2012).

Many measures of validity and reliability are available, such as technical error of measurement (TEM), intra-correlation coefficient (ICC), and coefficient of reliability (R). The use of these methods together allows a better estimate of the precision of anthropometric measurements (Geeta et al. 2009; Arroyo et al. 2010).

## 3.1 Rationale for the intra-observer reliability study

The reasons for conducting the reliability study arose from: 1) The importance of WC in assessing disease risk (Snijder et al. 2006). 2) The high potential error in WC measurement (Geeta et al. 2009). 3) The fact that studies I and II involved assessing the correlation between changes in anthropometric measures and risk factors for type 2 diabetes and cardiovascular diseases. Therefore, determining the accuracy, validity and precision of WC measurement was necessary to ensure differences in values between appointments were not to due to a high degree of error.

## 3.2 Objective

The objective of this study was to estimate the extent of intra-observer precision for waist circumference measurement.

## 3.3 Materials and methods

A sample of 10 volunteers participated in the study. The study was conducted by the same researcher who measured waist circumference on two different occasions. To limit the day-to-day variability in WC, participants made the two appointments in the same day.

Participants were asked to attend the first appointment at least 2 hours after having a meal. WC was measured twice. Participants were then asked to come back in a period of 30 minutes while having no food or drink during this time. Measurement of WC was also performed twice, and the average of each of the two measurements taken at the same time was calculated. The data collection was intended to hide the results immediately by the researcher after measurement 1 to minimize bias. Asking participants to come back after 30 minutes was mainly to avoid the researcher recalling the numbers.

Waist circumference was measured via the same metal measuring tape according to the protocol described in section 3.3.5.2. As this study only aimed to assess the intra-error of measurement of WC, no other measurements were taken.

The intra-examiner precision was determined by using four different estimates as suggested in the literature: TEM, the relative TEM (rTEM), ICC and coefficient of reliability (R) (Geeta et al. 2009; Arroyo et al. 2010).

a) The Technical error of measurement was calculated using the following formula:

$$TEM = \sqrt{\sum(d^2) / 2N}$$

TEM is defined as the standard deviation between measures, where d represents deviations and N is the number of volunteers measured. TEM is considered an index of accuracy, and the most commonly used indicator for measuring error and precision in anthropometry (Arroyo et al. 2010). The higher the precision of the measurement implies a lower TEM (Geeta et al. 2009). As far as we know, no

acceptable range of TEM for WC has been previously described in the literature. However, acceptable values of rTEM (see below) have been cited.

b) Relative TEM (rTEM) iss used to calculate the error as a percentage of the average of measures of WC:

$$rTEM = (TEM / VAV) * 100$$

VAV is the variable average value which corresponds to the mean of measurements for WC for volunteers. The acceptable variability for intra examiner TEM for WC has been estimated to be no more than 1.5 % for beginner anthropometrists, and no more than 1% for skilful ones (Perini et al. 2005).

c) The ICC is used to assess the degree of similarity between measurements (Geeta et al. 2009), by determining the variance between the samples. ICC is calculated on SPSS software (Bruton et al. 2000). It is suggested that R<0 implies no reliability, whereas values between 0.4 and 0.6 and 0.6 to 0.8 show moderate and substantial reliability, respectively. Values close to 1 indicate "nearly perfect reliability" (Geeta et al. 2009).

d) The coefficient of reliability is measured to assess variability between subjects without considering the measurement error. R can vary between 0 and 1. Values higher than 0.95 are considered highly precise (Arroyo et al. 2010). The coefficient of reliability is calculated in the following way:

$$R= 1- (TEM^2 / SD^2 )$$

SD (standard deviation) represents the total variance within subjects (Arroyo et al. 2010).

## 3.4 Statistical analysis

The statistical analysis was performed using SPSS version (version 17, SPSS Inc. Chicago IL). Level of significance was set at $p \leq 0.05$.

## 3.5 Results

Participants were all QMU staff and visitors. The mean age of the population was 35.3 ± 3.14 years. The mean difference between the values at time 1 and time 2 was small (-0.05 ± 0.88 cm). The BMI of this population ranged between 18 and 31 $Kg/m^2$. The mean and standard deviation was 83.69 ± 9.59 cm for measure 1 and 83.52 ± 9.89 cm for measure 2. The upper and lower limits of differences between the two measurements were between 1.15 and 0.15 cm, respectively.

TEM: The intra examiner TEM was estimated to be 0.41. As for rTEM, which involves calculating deviations for each for the 10 volunteers, results are shown in table 3.1.

Table 3.1: Relative TEM for study participants

| | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Measurement Time 1 | 64.75 | 87.5 | 77.25 | 92.35 | 93.85 | 85.2 | 90.15 | 73.65 | 79.8 | 92.35 |
| Measurement Time 2 | 63.6 | 87.75 | 77.4 | 92.4 | 94 | 85.5 | 89 | 73 | 79.95 | 92.62 |
| Deviations (d) | -1.15 | 0.25 | 0.15 | 0.05 | 0.15 | 0.3 | -1.15 | -0.65 | 0.15 | 0.27 |
| $d^2$ | 1.32 | 0.063 | 0.023 | 0.0025 | 0.023 | 0.09 | 1.32 | 0.42 | 0.023 | 0.073 |
| $\sum d^2$ | 3.36 | | | | | | | | | |
| TEM | 0.41 | | | | | | | | | |
| VAV | 83.6 | | | | | | | | | |
| rTEM % | **0.49** | | | | | | | | | |

*P: Participant, TEM: Technical error of measurement, rTEM: Relative TEM, VAV: Variable average value. For VAV, the average measurement of WC at two time points was calculated for each participant. The average for the 10 participants was then summed up and divided by 10.*

The determination of ICC via SPSS software yielded a coefficient of 0.998. The coefficient of reliability was estimated to be R = 0.99.

## 3.6 Discussion

The findings of this study showed that the estimate of error rTEM (0.49 %) indicate a high level of accuracy (<1% (Perini et al. 2005)). In addition, the consistency between measures was shown to be excellent, as demonstrated by ICC which was found to be 0.998. The degree of reliability was also shown to be high (r = 0.99). Results indicate a lower intra-observer degree of error than the study of Geeta et al. (2009) (TEM and rTEM were 0.77 and 0.91 respectively), which used the same protocol of WC measurement, and was conducted by inexperienced dietitians on 10 participants. The coefficient of reliability in the latter study (R = 0.999) was albeit similar to the current study.
In addition, the differences in WC measurement closely match with the results of a meta-analysis of 9 studies, which showed that the intra error of measurement for WC ranged between 0.7 cm and 9.2 cm. This large difference was mainly explained by the unskilled physicians who performed some of these studies (Verweij et al. 2012).

Furthermore, the difference between measurements taken at time 1 and time 2 in this study are below to what is considered clinically relevant, the latter term being defined as more than 5% decrease in WC over the short-term, and more than 3 % over the long term. This should correspond to a change of 3.5 cm for a WC of 70 cm, and 5.5 cm for a WC of 110 cm over the short term. Yet, the duration of the short and long terms were not specified. Therefore, considering that the values of WC generally range between 60 and 135 cm, clinically relevant short-term changes range between 3 - 6.8 cm, while clinically relevant long term changes range between 1.8-4.1 cm (Verweij et al. 2012). Consequently, this reliability study showed that it is possible to detect both short term and long term clinically relevant changes in WC despite the error of measurement. An important point to mention is that although it was reported that overweight and obese represent a higher error measurement of WC compared to normal weight individuals due to the difficulty in locating the bones, a larger decrease in waist circumference (for the same percentage) compared to normal

weight individuals is needed to detect a clinically significant change (Verweij et al. 2012).
As for the protocol of WC used in this study (the middle point between the lowest rib and the top of the hip bone), it has been stated in the meta-analysis of Verweij et al. (2012) that although 6 of the nine included studies included have used the same protocol, this way of measuring WC presents a higher degree of error compared to the measurement at the level of the lower rib. The latter site has been reported to be more easily measured. This is an important point to consider in the future in order to obtain a more accurate measurement of WC.

The findings of this intra-observer study were effective in determining the reliability of WC measurement when performed by the same researcher in subsequent studies. Findings also imply that the measuring tape used in this study is a reliable instrument to be used in the following two trials. Nonetheless, this study is limited by the impossibility of hiding the purpose of the study from the researcher, which might have resulted in a bias.

In conclusion, results suggest that measurement of waist circumference by the same researcher is accurate, as the intra-error of measurement in this study is minimal. It is then possible to perform this method to measure WC in studies I and II. Future studies should probably consider establishing a uniform protocol for the measurement of WC.

# CHAPTER 4: RESULTS AND DISCUSSION

## 4.1 Study I

### 4.1.1 Results

#### 4.1.1.1 Baseline and population characteristics

Advertisement via the university email generated the highest rate of response to the study followed by word-of mouth. Sixty-one volunteers completed the trial. The study flow is illustrated in Figure 4.1. Regarding ethnicity, 88.5% of participants were Europeans while the rest were Chinese (3.3%), middle Easterns (3.3 %), Africans (1.6%) and south/central Americans (1.6%) and 1.6% of participants were described as having other ethnicities. As for occupation, 86.9% were students and 13.1% were employed. Female participants represented 80.3% of the study population. One woman was postmenopausal. The baseline characteristics of the overall study population and split according to the type of intervention are presented in Table 4.1.

**Table 4.1: Baseline characteristics of the study population**

| | Study population N=61 | Placebo group N=30 | PRDC group N=31 | Difference between groups Significance (p= ) |
|---|---|---|---|---|
| | Mean ± SD | | | |
| Age (years) | 28.82 ± 8.89 | 28.13 ± 8.98 | 29.48 ± 8.89 | *0.48* |
| BMI ($Kg/m^2$) | 23.92 ± 4.17 | 24.08 ± 3.78 | 23.77 ± 4.57 | *0.4* |
| WC (cm) | 77.7 ± 10.52 | 76.83 ± 8.89 | 78.54 ± 11.98 | *0.17* |

*PRDC: Polyphenol-rich dark chocolate, WC: Waist circumference. Results are expressed as means ± SD. Data was analysed using an independent t-test.*

As shown in Table 4.1, no differences in baseline characteristics between the placebo and PRDC group were noted. In addition, there were no gender differences (p= 0.063) between the 2 groups. Based on the BMI classification, 22 participants

were overweight and 39 were normal weight. Among participants with BMI> 25 Kg/m$^2$ (N=22), 17 were overweight (BMI< 30 Kg/m$^2$) while the rest were obese. Three normal weight and 20 overweight participants had a high WC (classification of WC is presented in Figure 2.4), and were classified at increased risk of metabolic complications. BMI and WC seem to follow a linear model. There was a strong linear association between BMI and WC (r (Pearson's) = 0.913, p<0.001) (Figure 4.2).

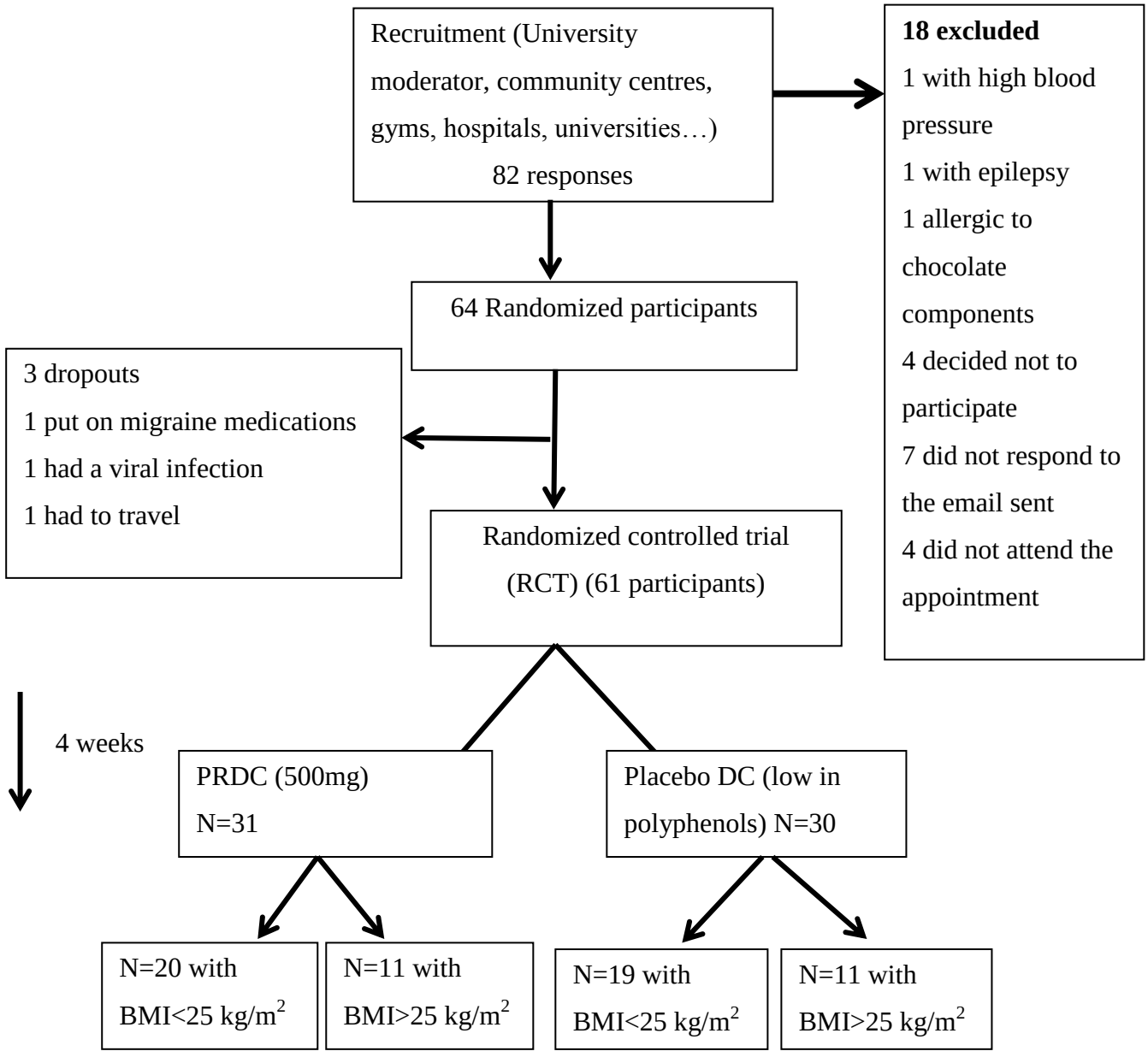



**Figure 4.1: Flow of Study I.**
*PRDC: Polyphenol-rich dark chocolate*

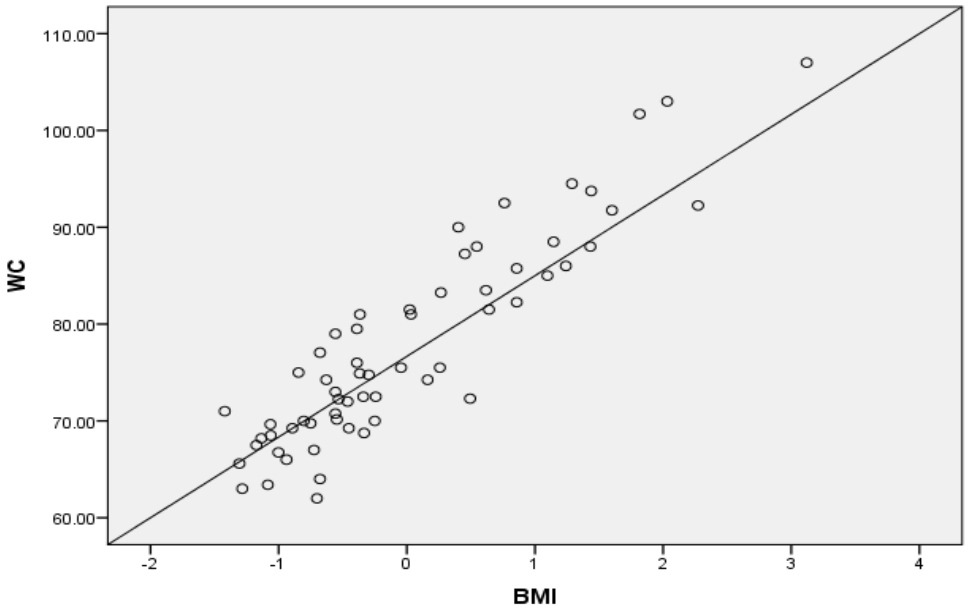



**Figure 4.2: Correlation between BMI and WC in Study I**

*WC: Waist circumference. WC (cm). BMI: values represent regression standardized predicted values. (r (Pearson's) = 0.913, p<0.001).*

Regarding supplement intake, only one participant reported having a usual intake of dietary supplements to increase physical capacity (chondroitin). Furthermore, 57.1% of volunteers reported having a family history of chronic diseases such as diabetes, hypertension and/or cardiovascular diseases, and 19 females mentioned taking contraceptives.

In relation to physical activity, 60.9% of the population mentioned practising more than 3 hours of exercise per week, and no significant differences in the baseline reported levels of physical activity (when converted to MET/ week) was noted between the placebo DC and the PRDC group (Placebo DC: 14.35 ± 14.01 MET/week; PRDC: 21.08 ± 17.09 MET/week, *p= 0.1*).

Four participants did not have their blood withdrawn due to difficulty in venepuncture. All participants had their blood pressure taken and they all provided saliva samples. However, five samples were excluded from the analysis due to the insufficient amount of saliva provided.

An independent t-test showed no differences in baseline measurements between placebo DC and PRDC groups regarding insulin (p= 0.5), glucose (p= 0.27), HOMA-IR (p= 0.41), QUICKI (p= 0.11), SBP (p= 0.96), DBP (p= 0.28) , serum lipids (TC (p= 0.7), TG (p= 0.98), HDL (p= 0.27) and LDL (p= 0.98)), hs-CRP (p= 0.59) and oxidised LDL levels (p= 0.45). In addition, there was no significant difference in the baseline levels between the 2 groups regarding cortisol (p=0.31), cortisone (p= 0.77) and 11- β HSD1 activity (p= 0.83) (Table 4.2). None of the variables violated the assumption of normality with the exception of cortisol, cortisone and the resultant cortisol/cortisone ratio (11-β HSD1). The latter parameters were then analysed using Wilcoxon and Mann whitney tests.

Table 4.2: Baseline levels of assessed parameters between placebo DC and PRDC groups

| | Placebo DC | PRDC |
|---|---|---|
| Insulin (µIU/ml) | 6.52 ± 3.83 | 5.83 ± 3.87 |
| HOMA-IR | 1.42 ± 0.89 | 1.23 ± 0.84 |
| QUICKI | 0.73 ± 0.15 | 0.86 ± 0.4 |
| Glucose (mmol/l) | 4.84 ± 0.37 | 4.7 ± 0.55 |
| SBP (mmHg) | 119.2 ± 11.85 | 119.03 ± 12.99 |
| DBP (mmHg) | 77.37 ± 7.71 | 75.35 ± 6.76 |
| TC (mmol/l) | 4.26 ± 0.79 | 4.17 ± 1.07 |
| LDL cholesterol (mmol/l) | 2.23 ± 0.8 | 2.23 ± 0.97 |
| HDL cholesterol (mmol/l) | 1.66 ± 0.35 | 1.56 ± 0.36 |
| TG (mmol/l) | 0.83 ± 0.32 | 0.96 ± 0.37 |
| Oxidised LDL (ng/ml) | 60.07 ± 16.66 | 63.68 ± 17.83 |
| Hs-CRP (mg/l) | 1.35 ± 0.97 | 1.5 ± 1.04 |
| Cortisol (nmol/l) | 3.34 ± 1.88 | 2.82 ± 1.49 |
| Cortisone (nmol/l) | 4.39 ± 2.4 | 4.5 ± 3.03 |
| 11-β HSD1 (cortisol/cortisone ratio) | 0.99 ± 1.06 | 0.79 ± 0.46 |

*HOMA-IR: Homeostasis modelling assessment-IR, QUICKI: Quantitative insulin sensitivity check index, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: Total cholesterol, LDL: Low density lipoprotein; HDL: High density lipoproteins, TG: Triglycerides, hs-CRP: high sensitivity C-reactive protein, 11 β-HSD1: 11-beta hydroxysteroid dehydrogenase 1. There were no significant differences in baseline levels of assessed parameters between the 2 groups (p> 0.05). Results are expressed as mean ± SD.*

#### 4.1.1.2 Haematological assessment

*a. Insulin, glucose levels, HOMA and QUICKI*

Results showed a significant effect of PRDC on insulin levels ($p< 0.001$), HOMA-IR ($p=0.003$), and QUICKI ($p< 0.001$), but no significant effect on glucose levels ($p= 0.16$). Participants in the PRDC group had a significant decrease in insulin levels (from 5.83 ± 3.87 µIU/ml to 4.66 ± 3.86 µIU/ml), HOMA-IR (from 1.23 ± 0.84 to 1.02 ± 0.85), and an increase in QUICKI (from 0.86 ± 0.4 to 1.98 ± 0.4) after four weeks. Interestingly, participants administered placebo DC had an increase in their insulin levels (from 6.52 ± 3.83 µIU/ml to 7.29 ± 4.52 µIU/ml, *p= 0.014*), HOMA-IR (from 1.42 ± 0.89 to 1.69 ± 1.07, *p=0.003*), and a decrease in QUICKI (from 0.73 ± 0.15 to 0.69 ± 0.79, *p=0.013*). Also, glucose levels increased at the end of the study in the placebo DC group by 0.44 ± 1.08 mmol/l ($p= 0.041$) (Figures 4.3 and 4.4 and 4.5).

However, the positive effect on insulin sensitivity in the PRDC group was not associated with an amelioration of β-cell function estimated by HOMA-β ($p= 0.49$). Also, no significant change in this indicator was noted in the placebo DC group ($p=0.7$) as a result of an increase in insulin and glucose levels.

As for the magnitude of association between markers of glucose and markers of insulin metabolism in the study population, pearson's correlation showed a strong association between changes in insulin and changes in HOMA-IR ($r= 0.904$, $p<0.001$) and QUICKI ($r= -0.502$, $p< 0.001$). However, changes in glucose levels were not correlated to post-pre differences in HOMA-IR index ($r=0.037$, $p= 0.79$), and QUICKI ($r= -0.2$, $p= 0.13$). These results suggest that changes in insulin resistance/sensitivity in this study were principally correlated to changes in insulin levels.

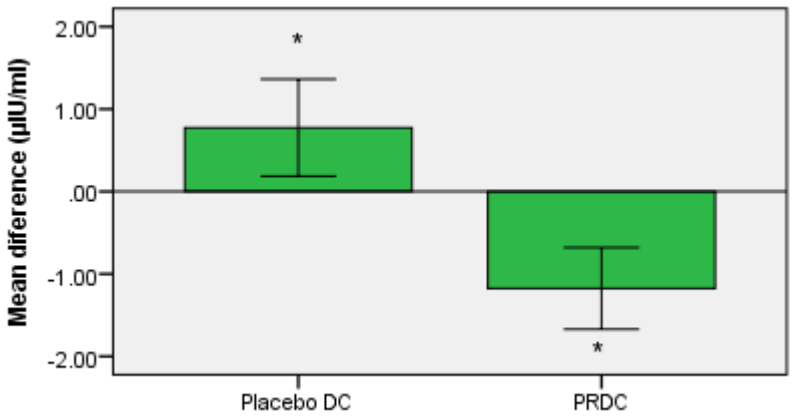



**Figure 4.3: Changes in insulin levels from baseline in the placebo DC and PRDC groups following the intervention**

** p<0.05.DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Data expressed as mean ± SEM. Bar charts represent the differences from baseline (post – pre) in the placebo DC and PRDC groups after 4 weeks. Data was analysed using paired-t-test.*

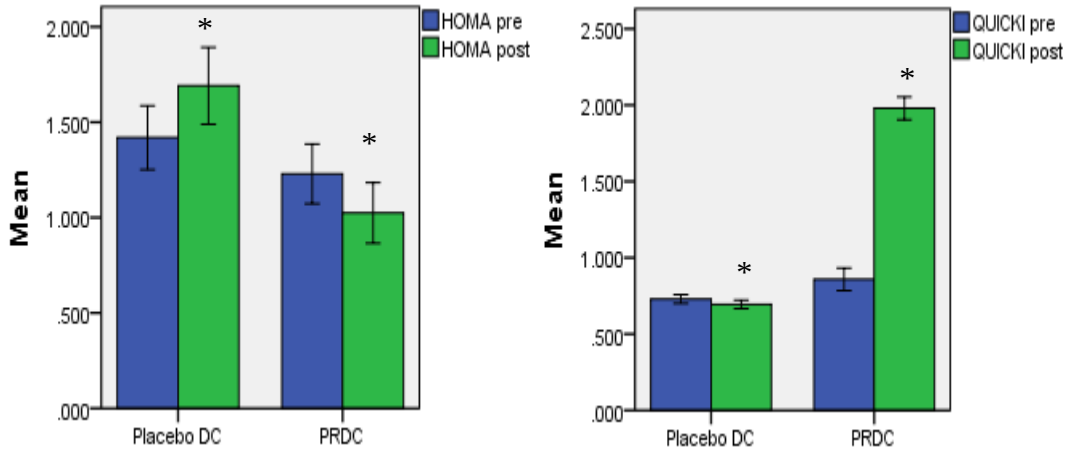



**Figure 4.4: Changes in QUICKI and HOMA-IR indexes following PRDC consumption compared to placebo**

** Significant difference from pre intervention (baseline), p < 0.05. HOMA-IR: Homeostasis modelling assessment-insulin resistance, QUICKI: Quantitative insulin sensitivity check index, DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Data was analysed using paired-t-test and expressed as mean ± SEM.*

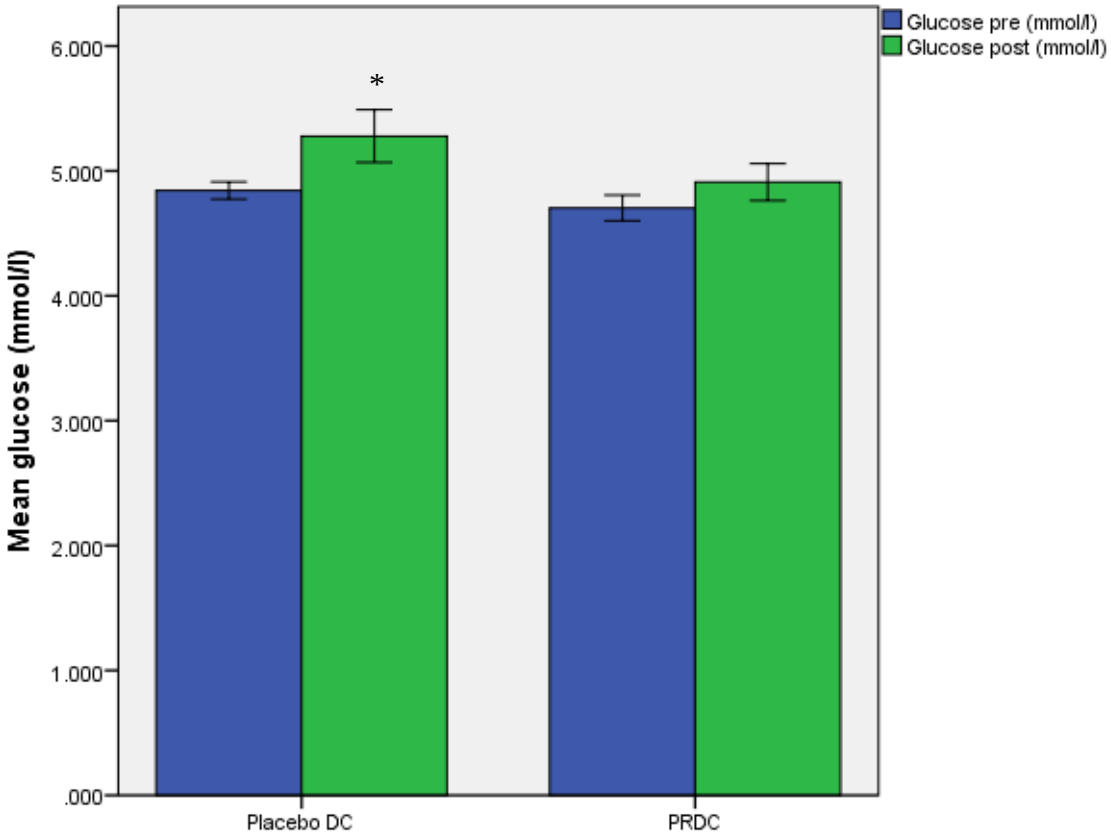



**Figure 4.5: Changes in glucose levels in the placebo DC and PRDC after 4 weeks.**

** Significant difference from pre intervention (baseline), p < 0.05. DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Results expressed as mean ± SEM. Data was analysed using paired-t-test.*

***b.*** *Lipid profile*

Results showed that TC, HDL and LDL levels did not significantly change throughout the intervention in both groups (p>0.05). However, there was a significant increase in TG levels in the placebo DC group. In the PRDC group, TG levels decreased but did not reach statistical significance (Table 4.3).

**Table 4.3: Changes in serum lipid levels from baseline following the intervention**

| Lipid levels (mmol /l) | Pre-PRDC Mean ± SD | Post-PRDC Mean ± SD | Δ (Post-PRDC - Pre-PRDC) | p | Pre-placebo Mean ± SD | Post-placebo Mean ± SD | Δ (Post-placebo - Pre-placebo) | p |
|---|---|---|---|---|---|---|---|---|
| TC | 4.17±1.07 | 4.23±0.98 | 0.059±0.76 | 0.68 | 4.26 ± 0.79 | 4.5 ± 1.15 | 0.24± 0.91 | 0.18 |
| LDL | 2.23±0.97 | 2.22±1.06 | -0.013±0.66 | 0.92 | 2.23 ± 0.8 | 2.35 ± 1.08 | 0.11 ± 0.73 | 0.43 |
| HDL | 1.56±0.36 | 1.64±0.34 | 0.08 ± 0.27 | 0.12 | 1.66 ± 0.35 | 1.73 ± 0.4 | 0.07 ± 0.24 | 0.12 |
| TG | 0.83±.0.46 | 0.69±0.23 | -0.15 ± 0.43 | 0.07 | 0.83 ± 0.32 | 0.96 ± 0.37 | 0.13 ± 0.23* | **0.008** |

**Significant difference from pre intervention (baseline), p< 0.05. DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, TC: Total cholesterol, LDL: Low density lipoprotein, HDL: High density lipoproteins, TG: Triglycerides. Data was analysed using paired t-test and expressed as Mean ± SD.*

*c. LDL oxidation levels*

A significant effect of treatment on oxidised LDL levels was only noted in the PRDC group (p= 0.042). Levels of oxidised LDL dropped from 63.68 ± 17.83 ng/ml to 57.03 ± 14.08 ng/ml after PRDC consumption, while in the placebo group, no significant change in these levels occurred (Baseline: 60.07 ± 16.66 ng/ml, post intervention: 61.92 ± 16.34 ng/ml, p= *0.42*) (Figure 4.6).

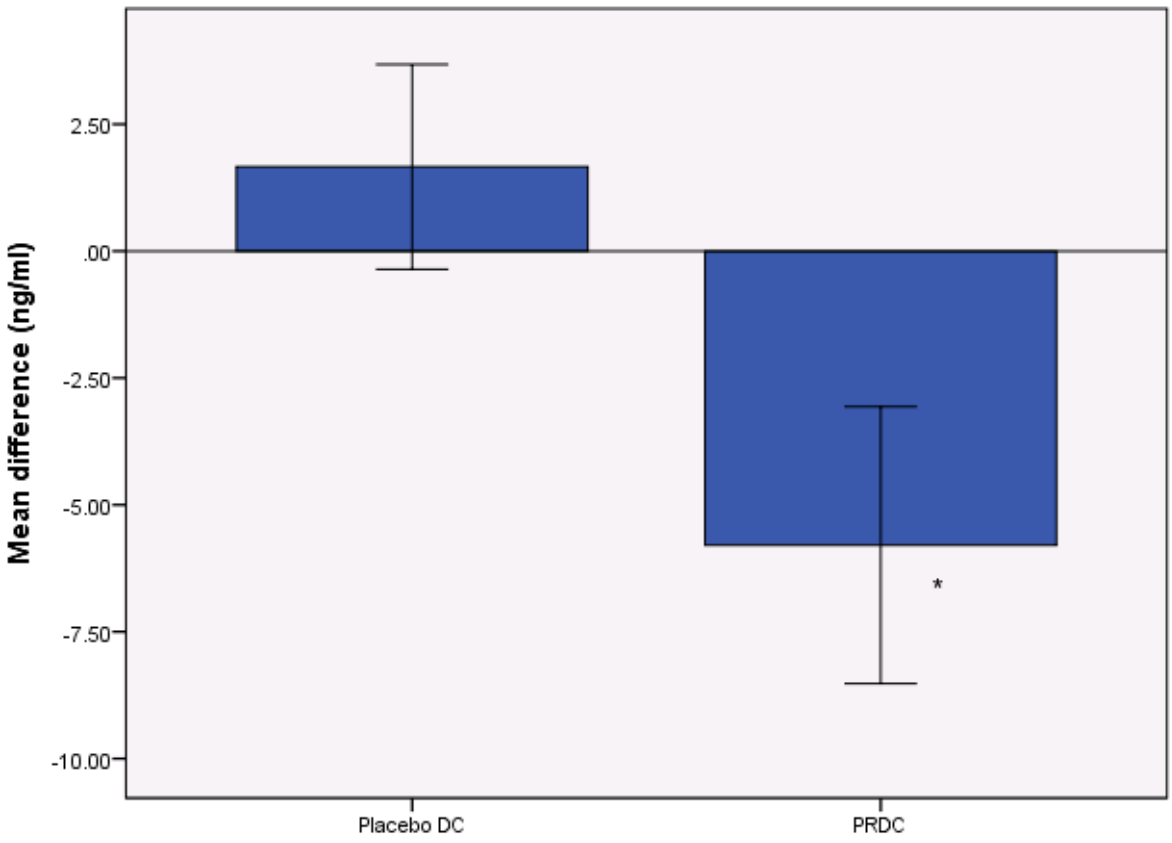



**Figure 4.6: Changes in LDL oxidation levels from baseline following the consumption of PRDC and placebo DC.**

**p<0.05. PRDC: Polyphenol-rich dark chocolate, LDL: Low density lipoproteins. Bar charts represent the differences from pre intervention (baseline) in the placebo DC and PRDC groups after 4 weeks. Results expressed as means ± SEM. Data was analysed using paired-t-test.*

*d. High sensitivity CRP (Hs-CRP)*

Results showed no significant changes in the inflammatory marker hs-CRP in both PRDC from baseline (from 1.35 ± 0.97 mg/l to 1.26 ± 0.88 mg/l; Δ= -0.46 ± 0.2 mg/l, *p= 0.32*), and placebo DC groups (from 1.5 ± 1.04 mg/l to 1.54 ± 1.12; Δ= 0.09 ± 0.8 mg/l, *p= 0.57*) after 4 weeks.

#### 4.1.1.3 Cortisol, cortisone levels, and the activity of the 11-β HSD1

Results showed no significant changes in cortisol and cortisone levels, and in the activity of the enzyme 11-β HSD1 (cortisol/cortisone ratio), following the consumption of placebo DC or PRDC group (Table 4.4).

**Table 4.4 : Changes in saliva cortisol and cortisone levels and in the activity of 11-β HSD1 from baseline in the placebo DC and PRDC groups**

| | Placebo (Mean difference ± IQR) | *p* | PRDC (Mean ± IQR) | *p* |
|---|---|---|---|---|
| Cortisol (nmol/l) | 0.33 ± 0.96 | *0.1* | 0.11 ± 0.54 | *0.27* |
| Cortisone (nmol/l) | 0.37 ± 1.17 | *0.22* | -0.77 ±2.05 | *0.34* |
| 11-β HSD1(Cortisol/ cortisone ratio) | -0.14 ± 0.12 | *0.44* | -0.2 ± 0.38 | *0.1* |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, 11 β-HSD1: 11-beta hydroxysteroid dehydrogenase 1. Data expressed as Mean difference (IQR: interquartile range). Changes from baseline were not significant in both groups after 4 weeks.*

#### 4.1.1.4 Blood pressure

There were no significant changes in blood pressure following the intervention. In the placebo group, no significant changes from baseline in SBP (pre: 119.2 ± 11.95 mmHg; post: 117.39 ± 9.98 mmHg, *p= 0.14*) or DBP (pre: 77.37 ± 7.71 mmHg; post: 77.13 ± 7.97, *p= 0.8*) were noted. Similarly, SBP (pre: 119.03 ± 12.99 mmHg, post: 119.07 ± 11.88 mmHg, *p= 0.97*) and DBP (pre: 75.35 ± 6.76 mmHg, post: 75.52 ± 6.3, *p= 0.88*) did not significantly change in the PRDC group after 4 weeks.

#### 4.1.1.5 Weight, BMI and waist circumference

Analysis of differences from baseline showed that weight, BMI and WC did not significantly change in the PRDC group, whereas four weeks of daily DC consumption increased weight in the placebo DC group (Table 4.5).

Table 4.5: Changes in anthropometric measurements in the placebo DC and PRDC groups following the intervention

| | Pre-PRDC Mean ± SD | Post-PRDC Mean ± SD | Δ (Post-PRDC - Pre-PRDC) | *p* | Pre- placebo Mean ± SD | Post-placebo Mean ± SD | Δ (Post-placebo - Pre-placebo) | *p* |
|---|---|---|---|---|---|---|---|---|
| Weight (Kg) | 70.26±16.04 | 70.25±16.32 | -0.01±0.9 | 0.98 | 66.16± 12.43 | 66.6± 12.54 | 0.44± 0.86* | **0.009** |
| BMI ($Kg/m^2$) | 23.77± 4.57 | 23.76±4.65 | -0.01±0.3 | 0.82 | 24.08±3.78 | 24.25±3.87 | 0.17 ±0.32* | **0.007** |
| WC (cm) | 78.54±11.98 | 78.75±11.8 | 0.21± 1.7 | 0.5 | 76.83±8.89 | 76.96±8.98 | 0.13 ±0.84 | 0.42 |

**Significant difference from pre intervention (baseline), p< 0.05. WC: waist circumference, DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Data analysed using paired t-test and expressed as Mean ± SD.*

#### 4.1.1.6 ANCOVA measures

In order to control for any potential baseline imbalances, and to identify significant differences between groups, ANCOVA measures were performed for the assessed parameters, with dependent variables being the post values, the covariates being the pre values, and the fixed factor being the type of intervention. Results showed significant differences between groups for HOMA-IR, QUICKI, Insulin, TG, oxidised LDL, BMI and weight, but not for waist circumference, glucose, blood pressure, hs-CRP, and other serum lipid levels. None of the above mentioned variables violated the assumption of heterogeneity (Levene's test > 0.05). ANCOVA results are shown in Table 4.6.

**Table 4.6: Differences between groups after adjusting for baseline values of assessed parameters in the placebo and PRDC groups**

| | | **Placebo DC** | | **PRDC** | | |
|---|---|---|---|---|---|---|
| | Adjusted pre mean value for both groups | Adjusted post mean value | 95% CI | Adjusted post Mean value | (95% CI | Difference between groups *p* |
| Glucose (mmol/l) | 4.77 | 5.23 | (4.88, 5.59) | 4.96 | (4.61, 5.3) | 0.27 |
| Insulin (µIU/ml) | 6.17 | 6.93 | (6.38, 7.5) | 5.0 | (4.46, 5.55) | **<0.001** |
| HOMA-IR | 1.32 | 1.6 | (1.44, 1.74) | 1.12 | (0.97, 1.27) | **<0.001** |
| QUICKI | 0.8 | 0.66 | (0.56, 0.76) | 2.01 | (1.9, 2.11) | **<0.001** |
| TC (mmol/l) | 4.22 | 4.46 | (4.16, 4.77) | 4.26 | (3.96, 4.57) | 0.35 |
| LDL (mmol/l) | 2.23 | 2.34 | (2.08, 2.61) | 2.22 | (1.96, 2.48) | 0.5 |
| HDL (mmol/l) | 1.61 | 1.7 | (1.6, 1.79) | 1.68 | (1.6, 1.77) | 0.8 |
| TG (mmol/l) | 0.83 | 0.96 | (0.86, 1.06) | 0.69 | (0.59, 0.78) | **<0.001** |
| Oxidised LDL (ng/ml) | 61.88 | 62.97 | (58.25, 67.69) | 55.98 | (51.26, 60.7) | **0.041** |
| Hs-CRP (mg/L) | 1.42 | 1.32 | (1.1, 1.54) | 1.48 | (1.26, 1.7) | 0.31 |
| SBP (mmHg) | 119.13 | 117.32 | (115.22, 119.43) | 119.13 | (117.06, 121.2) | 0.23 |
| DBP (mmHg) | 76.34 | 76.4 | (74.57, 78.22) | 76.23 | (74.44, 78.02) | 0.9 |
| Weight (Kg) | 68.24 | 68.71 | (68.39, 69.03) | 68.21 | (67.9, 68.52) | **0.031** |
| WC (cm) | 77.7 | 77.81 | (77.3, 78.3) | 77.92 | (77.44, 78.4) | 0.76 |
| BMI ($Kg/m^2$) | 23.92 | 24.1 | (23.98, 24.2) | 23.91 | (23.81, 24.02) | **0.028** |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, HOMA-IR: Homeostasis modelling assessment-insulin resistance, QUICKI: Quantitative insulin sensitivity check index, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: Total cholesterol, LDL: Low density lipoprotein; HDL: High density lipoproteins, TG: Triglycerides, hs-CRP: high sensitivity C-reactive protein, 11 β-HSD1: 11-beta hydroxysteroid dehydrogenase 1, WC: waist circumference, CI: Confidence interval. Data expressed as adjusted means (pre, post values) and 95% CI (lower bound, upper bound). Results represent the differences between PRDC and placebo DC groups after adjusting for baseline differences.*

As for glucocorticoid metabolism, the comparison of differences from baseline between the 2 groups (via Mann Whitney test) showed no significant difference in cortisol ($p = 0.58$), cortisone ($p= 0.11$) and 11-β HSD 1 activity levels ($p= 0.34$).

#### 4.1.1.7 Assessment of diet diaries and compliance

All participants returned the empty containers, and 5 participants reported forgetting or skipping the intake of the daily amount of chocolate for at least once, and no more than 3 times during the whole intervention period. The compliance was estimated to be 86.58 %. This was calculated by counting the returned plastic bags, and asking participants about the number of times they have missed the daily portion of chocolate over the study period (28 days). The percentage of compliance was calculated for each participant, and the average percentage was considered for the 61 participants.

Reported physical activity levels (MET/ week) did not significantly change throughout the intervention in the placebo group (Pre: 14.35 ± 14.01; MET/week post: 13.32 ± 14.76 MET/week, $p= 0.36$) and the PRDC group (Pre: 21.08 ± 17.09 MET/week; post: 20.37 ± 16.78 MET/week, $p= 0.54$).

All participants provided self-reported diet diaries. Paired-sample analysis on the Netwisp software (V3.0) showed no significant differences in energy and macronutrient intake following the intervention in both groups. Participants whether on a placebo DC or PRDC, maintained their usual diet throughout the study (Table 4.7). An independent t-test on Netwisp software was also carried out, and documented no significant differences in baseline energy and macronutrient intakes between the placebo group and the PRDC group ($p>0.05$).

**Table 4.7: Mean differences in energy and macronutrient intakes between the run-in period and week 3**

| | | Run-in period | Week 3 |
|---|---|---|---|
| Energy (Kcal) | *Placebo DC (N=30)* | 1799 ± 522 | 1746 ± 416 |
| | *PRDC (N=31)* | 1868 ± 564 | 1998 ± 644 |
| Carbohydrate (g) | *Placebo DC (N=30)* | 226.9 ± 64 | 208.3 ± 58 |
| | *PRDC (N=31)* | 233 ± 61 | 234 ± 62 |
| Protein (g) | *Placebo DC (N=30)* | 64 ± 22.2 | 63.2 ± 19.5 |
| | *PRDC (N=31)* | 73 ± 41.7 | 84 ± 49.5 |
| Fat (g) | *Placebo DC (N=30)* | 73.2 ± 32 | 73.4 ± 25 |
| | *PRDC (N=31)* | 72.6 ± 29.3 | 82.7 ± 34.3 |

*DC: dark chocolate, PRDC: polyphenol-rich dark chocolate. Results are expressed as mean ± SD. Differences were not significant (p>0.05). Data was analysed using paired t-test.*

#### 4.1.1.8 Subgroup analysis: Impact of BMI

To identify any impact of weight status on the outcomes, a subgroup analysis based on BMI was performed. Data was split according to the type of intervention (placebo DC or PRDC) and weight (BMI<25 Kg/$m^2$ and BMI>25 Kg/$m^2$). The baseline characteristics of the population stratified according to these criteria are presented in table 4.8.

**Table 4.8: Baseline characteristics of the study population split according to the study intervention and BMI**

| | | Placebo DC group N=30 (BMI<25 Kg/m$^2$, N=19; BMI>25 Kg/m$^2$, N=11) | PRDC group N=31 (BMI<25 Kg/m$^2$ N=20; BMI>25 Kg/m$^2$, N=11) |
|---|---|---|---|
| | | Mean ± SD | |
| Age (years) | BMI<25 Kg/m$^2$ | 24.95 ± 4.63 | 27.35 ± 7.34 |
| | BMI>25 Kg/m$^2$ | 33.64 ± 11.94 | 33.36 ± 10.39 |
| BMI (Kg/m$^2$) | BMI<25 Kg/m$^2$ | 21.64 ± 1.56 | 20.96 ± 1.66 |
| | BMI>25 Kg/m$^2$ | 28.3 ± 2.45 | 28.89 ± 3.58 |
| WC (cm) | BMI<25 Kg/m$^2$ | 71.21 ± 4.77 | 71.67 ± 5.48 |
| | BMI>25 Kg/m$^2$ | 86.55 ± 4.96 | 91.02 ± 10.37 |

*WC: waist circumference, DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Results are expressed as mean ± SD.*

Analysis via Mann-Whitney test showed a significant age difference between the 2 BMI categories in the group administered placebo DC (p=0.034), but not in the PRDC group (p=0.1).

Regarding other variables, in the placebo DC group, there was a significant difference in baseline levels of SBP (p= 0.012), DBP (p= 0.015), insulin levels (p= 0.021), HOMA-IR (p= 0.024), QUICKI (p= 0.024), as well as in TC (p= 0.031), HDL (p= 0.055) and LDL (p= 0.003)) levels between normal weight and overweight participants. All the parameters were higher in the overweight group, while HDL levels were lower. While in the PRDC group, there was a significant difference in glucose levels (p= 0.015), as well as in TC (p= 0.034), HDL (p= 0.055), LDL (p= 0.04), TG (p= 0.003) and hs-CRP (p= 0.013) levels between the two BMI categories (in the overweight group, all the parameters were higher, whereas HDL levels were lower).

Interestingly, participants whether normal weight or overweight did not significantly differ in self-reported baseline energy and macronutrient intake levels. In the placebo

group, no differences between the two BMI categories regarding energy (p= 0.72), protein (p= 0.43), fat (p= 0.91) and carbohydrate (p=0.72) were noted, similarly to the PRDC group (p= 0.46, p= 0.88, p= 0.21 and p= 0.56, respectively) (Table 4.9). Additionally, no significant differences in the baseline levels of physical activity (assessed by MET/week) were noted between the normal weight and the overweight category in the placebo DC (p = 0.16) and PRDC (p= 0.17) groups.

**Table 4.9: Differences in baseline energy and macronutrient levels between normal weight and overweight individuals in both placebo DC and PRDC groups**

| | | **Placebo DC** | **PRDC** |
|---|---|---|---|
| | | **Mean 95% CI** | **Mean 95% CI** |
| Energy (Kcal) | BMI<25 Kg/$m^2$ | 1848 (1548, 2147) | 1886 (1600, 2143) |
| | BMI>25 Kg/$m^2$ | 1719 (1474, 1965) | 1837 (1504, 2164) |
| Carbohydrates (g) | BMI<25 Kg/$m^2$ | 234 (199, 269) | 238 (210, 266) |
| | BMI>25 Kg/$m^2$ | 238 ( 179, 252) | 225 (181, 269) |
| Protein (g) | BMI<25 Kg/$m^2$ | 65.35 (58.07, 72.64) | 78.18 (61.96, 94.39) |
| | BMI>25 Kg/$m^2$ | 60.0 (51.71, 68.29) | 83.47 (67.9, 99.04) |
| Lipids (g) | BMI<25 Kg/$m^2$ | 72.98 (57.75, 88.2) | 73.17 (59.11, 87.22) |
| | BMI>25 Kg/$m^2$ | 73.59 (49.25, 97.93) | 71.56 (51.74, 91.39) |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, CI: Confidence interval. Results expressed as mean pre (95% CI: Lower bound, Upper bound).*

To determine differences in responses based on BMI, Mann Whitney test was performed. Mean differences (post – pre) were compared between the 2 BMI categories in each group. Results showed a significant impact of BMI on HOMA-IR only in the placebo group, while no significant other differences were noted. Results are shown in Table 4.10.

**Table 4.10: Differences in responses to intervention based on BMI in the placebo DC and the PRDC groups**

| | | Placebo DC | | PRDC | |
|---|---|---|---|---|---|
| | | **Mean diff (IQR)** | **p value** | **Mean diff (IQR)** | **p value** |
| Glucose (mmol/l) | BMI<25<br>BMI>25 | 0.27 (0.83)<br>0.73 (0.88) | 0.11 | 0.35 (0.7)<br>-0.04 (1.13) | 0.39 |
| Insulin (µIU/ml) | BMI<25<br>BMI>25 | 0.3 (1.04)<br>2.2 (3.21) | 0.07 | -1.35 (1.15)<br>-1.25 (1.62) | 0.42 |
| HOMA-IR | BMI<25<br>BMI>25 | 0.13 (0.39)<br>0.63 (0.89)* | **0.017** | -0.25 (0.47)<br>-0.29 (0.56) | 0.85 |
| QUICKI | BMI<25<br>BMI>25 | -0.3 (0.08)<br>-0.06 (0.06) | 0.24 | 0.96 (1.06)<br>1.24 (1.05) | 0.38 |
| TC (mmol/l) | BMI<25<br>BMI>25 | 0.9 (0.85)<br>0.05 (0.85) | 0.35 | 0.17 (1.25)<br>0.01 (0.7) | 0.73 |
| LDL (mmol/l) | BMI<25<br>BMI>25 | 0.25 (1.14)<br>-0.02 (0.78) | 0.7 | -0.017 (1.32)<br>-0.11 (0.59) | 0.44 |
| HDL (mmol/l) | BMI<25<br>BMI>25 | 0.16 (0.49)<br>-0.04 (0.15) | 0.25 | 0.09 (0.28)<br>0.04 (0.37) | 0.58 |
| TG (mmol/l) | BMI<25<br>BMI>25 | 0.13 (0.4)<br>0.91 (1.37) | 0.59 | -0.07 (0.4)<br>-0.31 (0.55) | 0.13 |
| Hs-CRP (mg/L) | BMI<25<br>BMI>25 | 0.9 (0.97)<br>0.54 (1.32) | 0.56 | 0.25 (1.02)<br>0.32 (1.2) | 0.35 |
| Oxidised LDL (ng/ml) | BMI<25<br>BMI>25 | 0.9 (21.0)<br>-3.59 (40.75) | 0.58 | -0.25 (16.69)<br>-9.82 (27.29) | 0.17 |
| SBP (mmHg) | BMI<25<br>BMI>25 | 0.04 (10)<br>-4.78 (12.5) | 0.09 | -0.54 (0.7)<br>0.7 (6.3) | 0.22 |
| DBP (mmHg) | BMI<25<br>BMI>25 | 0.67 (8.0)<br>-0.44 (8.0) | 0.87 | 0.62 (5.5)<br>-1.3 (6.5) | 0.55 |
| Cortisol (nmol/L) | BMI<25<br>BMI>25 | 0.26 (1.35)<br>0.12 (1.33) | 0.32 | -0.21 (0.76)<br>0.48 (0.39) | 0.46 |
| Cortisone (nmol/l) | BMI<25<br>BMI>25 | 0.28 (1.26)<br>0.05 (1.47) | 0.96 | -1.32 (2.59)<br>-0.2 (1.72) | 0.38 |
| Cortisol/Cortisone ratio | BMI<25<br>BMI>25 | 0.78 (0.21)<br>-0.22 (0.34) | 0.22 | 0.12 (2.54)<br>0.54 (1.26) | 0.64 |
| Weight (Kg) | BMI<25<br>BMI>25 | 0.44 (1.05)<br>0.52 (1.6) | 0.3 | -0.2 (1.53)<br>0.32 (1.42) | 0.47 |
| WC (cm) | BMI<25<br>BMI>25 | 0.13 (1.0)<br>0.25 (0.75) | 0.29 | 0.81 (0.68)<br>-0.14 (1.98) | 0.24 |
| BMI ($Kg/m^2$) | BMI<25<br>BMI>25 | 0.12 (0.41)<br>0.24 (0.48) | 0.36 | -0.05 (0.44)<br>0.059 (0.21) | 0.09 |

**Significant difference in the response to intervention between the normal weight and overweight population in the placebo group, $p<0.05$. DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. HOMA-IR: Homeostasis modelling assessment-insulin resistance, QUICKI: Quantitative insulin sensitivity check index, SBP: systolic blood pressure, DBP: diastolic blood pressure, TC: Total cholesterol, LDL: Low density lipoprotein; HDL: High density lipoproteins, TG: Triglycerides, hs-CRP: high sensitivity C-reactive protein, 11 β-HSD1: 11-beta hydroxysteroid dehydrogenase 1, WC: waist circumference. Results are expressed as mean difference (IQR: Interquartile range).*

For HOMA-IR, Wilcoxon test was performed while splitting the placebo group based on BMI and type of intervention. Analysis showed that the increase in HOMA-IR was only in the overweight group (p= 0.017), while it was not affected in the normal weight group (p= 0.11) following placebo DC consumption.

#### 4.1.1.9 Qualitative data

Data regarding the acceptability of treatment were collected at the end of the intervention. Most of participants (. 65.6 %) found the dark chocolate consumption very acceptable, and 29.5 % found it somewhat acceptable, while the rest (4.9%) found it unacceptable. Interestingly, splitting participants whether they consumed PRDC or placebo with regards to acceptability, it was found that placebo DC was more acceptable than PRDC (Figure 4.7).

The reasons for unacceptability (or for the decrease in the tolerance for the experimental chocolate) stated were mainly the dense colour of the chocolate which made it less appealing (1 participant in the PRDC), the bitterness and intense flavour (3 participants in the PRDC) and the texture (2 participants: 1 in the placebo DC group and 1 in the PRDC group). The latter participant stated that the consumption of drops instead of a bar gives the impression of taking a medicine and consuming a lot of chocolate. Other participants found the chocolate unacceptable because of its side effects: acne outbreaks (one participant in the placebo DC group), headache (one participant in the PRDC group), nausea, bloating, winds, stomach pain (one participant in the PRDC group). These complaints, although noteworthy, did not result in any dropout. As for other comments mentioned by volunteers, one participant stated that DC sometimes tended to decrease his appetite for sweets after lunch, but to increase his cravings on other occasions. Five participants found it impractical to consume this amount of chocolate everyday, and one participant preferred a higher percentage of cocoa. Also, 72.1% of participants mentioned that they will continue consuming dark chocolate frequently.

Lastly, only 52.5% of participants were able to identify the assigned chocolate type, suggesting that the perception did not affect the outcomes.

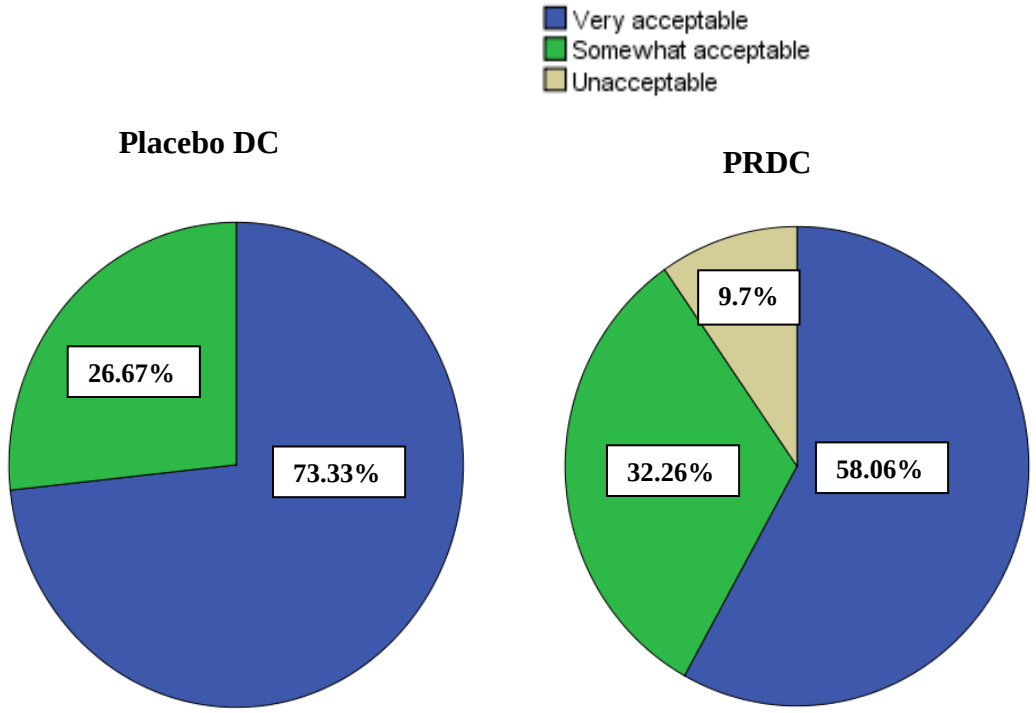



**Figure 4.7: Acceptability of treatment based on the type of intervention**
*DC: dark chocolate, PRDC: Polyphenol-rich dark chocolate*.

In summary, the results of study I showed that PRDC reduced fasting insulin, HOMA-IR, oxidised LDL levels, and increased QUICKI. On the other hand, placebo DC increased fasting insulin, HOMA-IR, glucose, TG and levels, and reduced QUICKI. Stratification based on BMI factor did not affect the parameters, with the exception of HOMA-IR, which only increased in overweight participants. The consumption of placebo DC was claimed to be more acceptable than PRDC.

### 4.1.2 Discussion

The principal aim of this study was to determine the effect of DC rich in polyphenols on insulin sensitivity in the adult population. To improve the reliability of the results, the heterogeneity was limited by adhering participants to eligibility criteria, and by avoiding confounding factors such as medications, supplements containing high doses of antioxidants, smoking, heavy alcohol consumption, and regular cocoa/dark

chocolate consumption. In addition, the two types of chocolate were matched for all components (theobromine, caffeine, fibre and minerals (like potassium or magnesium)) except for polyphenol content. This excluded potential confounding effects resulting from constituents other than polyphenols. For instance, an animal study showed that theobromine decreased TG, LDL and total cholesterol, and increased HDL in rats (Eteng and Ettarh 2000). This was supported by a human study which reported a lowering effect of theobromine (850 mg) on LDL, and an increasing effect on HDL following 2 weeks of daily consumption (Neufingerl et al. 2013). Caffeine was related to an increase in fat oxidation in humans (Acheson et al 2004), and magnesium was claimed to have hypotensive effects (Jee et al. 2002).

*Insulin and glucose metabolism*

This study showed that PRDC supplementation significantly decreased insulin levels (by 1.17 ± 1.34 µIU/ml) and improved insulin resistance/sensitivity expressed by a decrease in HOMA-IR (by 0.2 ± 0.34), and an increase in QUICKI (by 1.12 ± 0.74) in adults. The improvement in insulin sensitivity was in accordance with the results of the studies of Grassi et al. (2005), Davison et al. (2008) and Al Moosawi et al. (2012), conducted on participants with no history of diabetes, hypertension or CVD. While Davison et al. (2008) included only overweight participants, and Al Moosawi et al. (2012) noted a beneficial effect merely in the overweight and obese population, the results match with the outcomes of Grassi et al. (2005), and showed a significant amelioration in IR in normal weight and overweight adults. Yet, the use of two types of DC in study I (that looked almost identical) presents advantages over the study of Grassi et al. (2005), which used white chocolate as a placebo. This eliminated a potential expectation bias resulting from an unblinded study.

The results also correspond with the findings of a meta-analysis which reported that cocoa/DC supplementation induced a significant decrease in insulin levels (-2.65 µIU/ml; 95% CI: -4.65, -0.65) and HOMA-IR (-0.67 µIU/ml; 95% CI: -0.98, -0.36) (Hooper et al. 2012). Compared to the meta-analysis, the magnitude of decrease in insulin levels and HOMA-IR was lower in this study. This might be attributed to the fact that the meta-analysis included studies with patients, who might have higher

levels of metabolic complications (Silver et al. 2007), and thus might be more affected by the cocoa/chocolate intervention (Moosawi et al. 2012).

The favourable effects on insulin sensitivity might be attributed to the increase in the availability of NO (Grassi et al. 2005), and its subsequent effect on improving insulin signalling pathways (Bergandi et al. 2003). However, since there was no simultaneous change in blood pressure, the mechanism might not have involved an improvement of endothelial dysfunction, which in turn decreases insulin and blood pressure (Grassi et al. 2005). This suggests that PRDC might mediate IR through mechanisms other than the NO pathway. However, Egan et al. (2010) stated that the increase in NO bioavailability and vasodilatation do not imply an effect on blood pressure, unless this was accompanied by a decrease in renal pressure threshold for sodium hemostasis. Consequently, an improvement in insulin sensitivity might occur through a NO-mediated effect without affecting blood pressure. Nevertheless, NO levels/NOS enzymatic activity were not measured in this study, thus no definite conclusions regarding the mechanisms involved can be made. The decrease in carbohydrate absorption by cocoa polyphenols is also a suggested mechanism for the results noted. This includes the inhibition of digestive enzymes (Tadera et al. 2006), and/or intestinal glucose transporters (Cermak et al. 2004; Chen et al. 2007). Moreover, the implication of SCFA (the result of fermentation of undigested carbohydrates in the colon) in improving insulin sensitivity through increasing GLP-1 (which increases β-cell secretion and inhibits gastric emptying) can be proposed. Lastly, since HOMA- β did not significantly change in the PRDC group, this did not lead to an improvement of β-cell function. Yet, the limitations of this test remain in estimating pancreatic β-cell function under non stimulated conditions (Cobelli et al. 2007). Therefore, this test did not identify any possible amelioration in β-cell function in a non fasting state.

However, it is not clear which fraction of polyphenols was responsible for improving IR. Although epicatechin was previously attributed most of the IR lowering properties (Rutter 2001; Xuelin et al. 2001), procyanidins were recently suggested to have the greatest countering effect on insulin resistance among flavanols (Dorenkott

et al. 2014). The amount of procyanidins supposed to improve IR was proposed to be equivalent to 2.03 mg/Kg in humans (Dorenkott et al. 2014). Determining the efficacy of this considerably low dose of procyanidins in lowering insulin resistance might have an important implication. This could help making future recommendations based on lower amounts of chocolate, and consequently lower energy content. This could also reduce the adverse effects resulting from the intake of a high quantity of polyphenols. Studies that compare the effect of cocoa liquor to doses of flavanols monomers and procyanidins might help in elucidating the implication of each flavanol fraction in insulin metabolism. In addition, as quercetin was suggested to be implicated in decreasing hyperinsulinemia (Li et al. 2013), it is not known whether the amount of quercetin in the experimental chocolate was sufficient to induce such effects. In this study, the quantity of quercetin in PRDC was not assessed, but it is known that the quercetin dose was less 100 mg in the 20g daily portion. Studies looking at the amount that could be involved in the favourable effects on insulin sensitivity might need to be considered. Notably, a decrease in IR resulting from a combined effect of different fractions of flavonoids could also be proposed.

An important point to mention is that the hypoinsulinemic effect of cocoa polyphenols in this study corresponds to studies in the literature that found similar outcomes (Grassi et al. 2005; Davison et al. 2008), but contradicts other studies that showed an increase in insulin levels following cocoa/DC administration (Rutter 2001; Grassi et al. 2008; Yamashita et al. 2013). Although this appears confusing, it was suggested that the effect of these polyphenols on insulin and carbohydrate metabolism depends on the context, which results in different mechanisms of action of polyphenols. For instance, following carbohydrate ingestion, the increase in insulin secretion occurs through the stimulation of GLP-1 by flavanols, resulting in a decrease in glucose levels (Dorenkott et al. 2014). On the other hand, the effect of flavanols on the improvement of insulin signalling pathways and the decrease in insulin needs might lead to a decrease in fasting insulin levels (Grassi et al. 2005; Davison et al. 2008). These clarifications might be helpful in designing studies that aim to assess the increasing or the lowering effect of these flavonoids on insulin levels.

Unlike the studies held by Al Moosawi et al. (2010; 2012), who used the same type and daily amount of chocolate, this study showed no significant effect of PRDC on glucose levels ($p= 0.16$). The difference in the results might be attributed to some factors such as the adoption of a crossover study design in the studies of Al Moosawi et al. (2010; 2012). This might have resulted in a higher treatment effect than a parallel design, due the fact that crossover studies could be unblinded because of a carry-over effect (Feys et al. 2012). Moreover, it was noted that the non significant effect on glucose levels could be attributable to the study duration. A meta-analysis of 11 trials showed that studies lasting less than 3 weeks were the ones that showed a decrease in blood glucose levels, due to a higher compliance compared to longer term studies. Other factors, such as the dose of epicatechin used could not justify the non significant effects obtained in this study, since the dose that was shown to exert a lowering effect on glucose levels ranged between 50-100 mg/day (Hooper et al. 2012). In Study I, PRDC contained 85 mg of epicatechin. Furthermore, the results might be attributed to the fact that the decrease in blood glucose levels following PRDC consumption are mostly exhibited by a decrease in postprandial glucose levels. This was previously shown in the studies of (Gutiérrez-Salmeán et al. (2014), Grassi et al. (2008) and Al Jalil et al. (2008). This effect on postprandial glucose levels could be attributed to the procyanidin fraction (particularly oligomers) in cocoa. In fact, due to their low absorption, procyanidins might exert direct effects on glucose metabolism in the gastrointestinal tract, through several factors such as inhibiting carbohydrate absorption and decreasing the degradation of GLP-1. However, their effects on fasting glucose levels might be less effective (Dorenkott et al. 2014). Fractioning cocoa flavanols based on their degree of polymerisation is important to elucidate the mechanisms of action of these flavanols. In addition, measuring postprandial glucose levels might be important to identify the inhibitory effect of cocoa polyphenols on carbohydrate digestion and absorption.

As a result, it could be suggested that PRDC (with 500 mg of polyphenols) exerts its beneficial effects on insulin metabolism by decreasing insulin levels and insulin resistance without affecting glucose levels and β-cell function. These findings are particularly important because of the role of IR in the metabolic syndrome (Blaha

and Elasy 2006), and its major role in increasing arterial stiffness and atherosclerosis (Webb et al. 2010). This study demonstrated that PRDC might have a future implication in type 2 diabetes, and might constitute a further step towards integrating this snack into dietary programs targeted to prevent this chronic disease.

*Blood pressure*

The current study did not lead to a significant change in SBP or DBP following the consumption of PRDC. The results did not match with the outcomes of the studies of Al Moosawi et al. (2010; 2012), in which the same amount of DC and polyphenols were used, and a significant decrease in SBP and DBP was noted. Yet, the second study indicated a decrease in DBP only in the overweight population. This difference might be mainly attributed to the different study design as reported by the meta-analysis of Ried et al. (2010) on studies with cocoa and DC. This meta-analysis stated that crossover studies had a more pronounced effect on SBP and DBP than parallel studies. This supports the idea of a higher treatment effect noted in crossover studies compared to parallel studies, as stated by Feys et al. (2012).

In addition, other factors such as the duration of the study might have led to such results. Two meta-analyses reported that the reduction in blood pressure occurred in studies lasting 2 weeks but not in studies lasting longer than this period (Ried et al. (2010; 2012). The reason might be attributed to the adaptation to the quantity of polyphenols, thus providing an explanation for the results obtained by Taubert et al. (2007), who used a small amount of polyphenols (30 mg) in the intervention group, and noted a significant decrease in blood pressure (SBP: $-2.9 \pm 1.6$ mmHg and DBP: $-1.9 \pm 1.0$ mmHg $p<0.001$) after 18 weeks. In the current study, blood pressure was not assessed at 2 weeks interval; therefore, it was not possible to evaluate the adaptation effect to the daily dose of 500 mg of polyphenols over time.
On the other hand, it was stated that the dose of flavanols supposed to exert favourable effects on blood pressure might need to be high. In the 6-week study of Davison et al. (2010), the dose of 1052 mg of flavanols was the only amount effective in decreasing SBP and DBP, while the doses of 33, 372 and 712 mg did not induce such effect (in the current study, the dose of flavanols in PRDC was 400 mg).

This finding contradicts the idea of the adaptation to the quantity of polyphenols and the trial of Taubert et al. (2007). However, it is important to mention that the latter study was limited by the use of white chocolate in the placebo group. Therefore, the decrease in blood pressure might have been due to individual expectations and influence from peers, rather than to the effect of the small polyphenol dose (Egan et al. 2010).

Nevertheless, the results of Study I correspond with one particular finding of the meta-analysis of Ried. (2012), which performed a subgroup analysis based on blood pressure status, and documented no significant effects of cocoa polyphenols on BP in normotensive participants (SBP/DBP less than 120/80 mmHg) (Ried et al. 2012). Other meta-analyses that documented a decrease in SBP and or DBP mostly included normotensive and hypertensive participants (Desch et al 2010; Ried et al. 2010; Hooper et al. 2012), and did not stratify studies according to the above mentioned criterion.
Therefore, findings of this study suggest that PRDC might not have a lowering effect on blood pressure in normotensive individuals. To explore further, the original idea of the preventive effect of cocoa on blood pressure, which originated from the Kuna Indians living in remote islands might be misleading. In fact, the increase in blood pressure in this population upon migration to urban areas might not be only attributed to the decrease in cocoa consumption, but also to the change in their overall diet. The indigenous group of Indians used to consume four times as much fish, and twice as much fruit than the ones living in the rural areas (McCullough et al. 2006). Also, the increase in weight and obesity resulting from the adoption of an unhealthy diet, might have led to an increase in blood pressure in this population (Hall et al. 2001). Hence, the healthy levels of blood pressure in the indigenous Indians might have been a combination between a healthy diet and a healthy weight, rather than a particular contribution of cocoa.

Furthermore, it was stated that the potential effect of cocoa/DC on blood pressure could possibly be attributed to components other than polyphenols. These components were responsible for the blood pressure lowering effects in previous

studies. For instance, a high dose of theobromine (979 mg) in flavanol-rich cocoa was effective in decreasing SBP in 42 healthy individuals, while no effect with a low theobromine flavanol-rich cocoa (106 mg of theobromine) was noted (Van den Bogaard et al. 2010). The PRDC in study I contained 150 mg of theobromine, which was less likely to exert an effect on blood pressure.

Conversely, studies on hypertensives mostly showed a lowering effect on cocoa/DC on blood pressure (Taubert et al. 2003; Grassi et al. 2008; Davison et al. 2010). Although unclear, the mechanisms might include the inhibition of ACE by cocoa polyphenols (Katz et al. 2000; Actis-Goretta et al. 2003). Furthermore, as stated by Jia et al. (2010), patients with CVD diabetes and/or hypertension have in common some metabolic disorders such as inflammation, IR, dyslipidemia, and oxidative stress. Hence, by targeting inflammation and oxidative stress, cocoa polyphenols might help in decreasing blood pressure. This might provide an explanation for the blood pressure lowering properties of cocoa polyphenols in hypertensives. Therefore, it could be suggested that PRDC might help in normalizing high blood pressure levels in hypertensives, while it might possibly not affect normal blood pressure levels. Further long term studies are needed to elucidate these effects.

*Glucocorticoid metabolism*

This study did not note a significant change in cortisol and cortisone levels, and in the activity of 11-β HSD 1 enzyme. Results matched with the results of the studies undertaken by Al Moosawi et al. (2010; 2012), which showed no significant effects of PRDC on cortisol metabolism. However, findings did not correspond with the results of the study of Martin et al. (2009), which noted an improvement in glucocorticoid metabolism following two weeks of DC consumption. Results did not also correspond with other studies that reported a decrease in cortisol levels following polyphenol supplementation (Lee et al. 1996; Arion et al. 1997; Zhu et al. 2012), as well as a decrease in the activity of the enzyme 11 β-HSD1 (Hindzpeter et al. 2014). This might be explained by the fact that polyphenols other than flavanols might favourably affect cortisol metabolism. Indeed, studies that mostly reported favourable effects on cortisol metabolism included tea polyphenols (Zhu et al. 2012),

grape juice polyphenols (Lee et al. 1996) or chlorogenic acid (Arion et al. 1997), known to have a different composition than polyphenols in chocolate.

Cortisol was shown to be related to an increase in insulin levels. In diabetic animals, the inhibition of 11 β-HSD1 was associated with a decrease in glucose, insulin levels and HOMA-IR (Tomlinson et al. 2008). The fact that the activity of this enzyme did not significantly change, while HOMA-IR and insulin levels decreased in the PRDC group did not explain the findings. Nonetheless, it might be suggested that other confounding factors have not been controlled in the placebo and PRDC groups. For instance, a high fat intake is known to affect cortisol metabolism by increasing HPA activity (Drake et al. 2005), and a high sodium intake might increase urinary free cortisol levels (Chamarthi et al. 2007). Knowing that the analysis of diet diaries did not show a significant increase in fat intake during the intervention, results might be confounded by underreporting. In addition, it was not possible to obtain an accurate overview of sodium intake through the analysis of diet diaries, due to the fact that the food composition tables (2010) lacked many common foods. Hence, no accurate results could be obtained. Further studies evaluating the effect of PRDC on cortisol/cortisone levels are needed before concluding the lack of a lowering effect of PRDC on cortisol levels and 11 β-HSD1 activity.

*Lipid profile*

No significant changes in serum lipids levels were noted following the consumption of PRDC. The findings of this study fairly matched with many other studies on cocoa/DC supplementation and lipid profile (Mathur et al 2002; Engler et al 2004; Grassi et al 2005; Taubert et al 2007; Al Moosawi et al. (2010; 2012); Neufingerl et al. 2013). However, results did not correspond with other trials which reported a beneficial effect on serum lipid levels (Mursu et al. 2004; Fraga et al. 2005; Baba et al. 2007b; Balzer et al. 2008). The meta-analysis of Jia et al. (2010) explained the non significant effect on lipid profile by the fact that most of the recruited participants do not have history of CVD, diabetes or hypertension, which made cocoa/DC unlikely to induce significant changes in lipid levels. In fact, oxidative stress, which is common in individuals with cardiovascular risk, can lead to an

increase in cholesterol biosynthesis. The latter can be counteracted by antioxidants (Gesquière et al. 1999) such as cocoa polyphenols. In Study I, participants had normal cholesterol levels (less than 5 mmol/l). Therefore, the baseline health status of individuals might have led to the non significant results obtained.

In relation to the daily amount of polyphenols, the dose of 500 mg might have led to the non significant effects in this study. A meta-analysis reported that a daily amount of polyphenols less than 260 mg was effective in inducing a favourable effect on serum lipid levels (Jia et al 2010), while a higher dose did not cause such effect. The meta-analysis of Tokede et al. (2011) noted a saturation effect with a dose of 500 mg daily. It was suggested that a high amount of PPs could counteract their beneficial effects on serum lipids (Jia et al. 2010). The study duration might also have had an influence on the outcomes. Both meta-analyses of Tokede et al. (2010) and Jia et al. (2010) showed that studies lasting around 2 weeks demonstrated a significant effect on lipid profile, while this was not the case for studies of longer duration (more than 4 weeks). This was also concluded by the meta-analysis of Hooper et al. (2012) which documented that studies with duration less than 3 weeks were the only ones effective in decreasing HDL, LDL and total cholesterol. Tokede et al. (2011) explained the findings by a decrease in compliance encountered in longer term studies. It is worth mentioning that, although non significant, there was a trend towards a decrease in LDL levels in the PRDC group in study I, along with an increase in LDL in the placebo group. Testing the parameters every 2 weeks would have been effective in determining a potential decrease in adherence arising from daily PRDC consumption for a period longer than 2 weeks.

Furthermore, the non significant effects on lipid profile might be due to the fact that placebo DC and PRDC were controlled for theobromine. A high dose of theobromine (850 mg) increased HDL and decreased LDL levels in humans, while a lower dose of theobromine (150 mg) did not induce a significant effect (Neufingerl et al. 2013). Studies that did not note a significant effect on lipid profile (Grassi et al. 2005a; 2005b) had a low amount of theobromine (170 mg), which was similar to the current study (150 mg).

Lastly, some of the studies that showed an improvement in lipid profile used higher levels of fat, and consequently higher levels of cocoa butter than study I. Indeed, cocoa butter contains 33 % of oleic acid (USDA 2011a) which was reported to improve lipid profile (Corti et al. 2009). The amount of fat in the experimental PRDC in Study I was 7.44g, compared to 11.5 g, 21.3 g. and 75 g in the studies of Baba et al. (2007b), Fraga et al. (2005), and Mursu et al. (2004), respectively. The latter studies were the ones that showed a beneficial effect of cocoa/DC on lipid profile. However, the implication of oleic acid in DC in improving serum lipid levels can be contradicted by the prooxidant effects of cocoa butter that have been previously reported. These prooxidant effects are due to the unsaturated fats of cocoa butter (40% of total fats), which are highly prone to oxidation. This might counteract the effect of oleic acid on improving serum lipid profile (Vinson et al. 2006).

Based on the arguments exposed above, it is then possible to suggest that PRDC does not constitute a source of prevention against dyslipidemia. Further long term studies measuring serum lipid levels at different intervals (every 2 weeks), and with different doses of polyphenols are needed to elucidate the effect of PRDC on lipid profile in participants with no CVD.

*LDL oxidation levels*

This study showed that 500 mg of polyphenols in DC exert lowering effects on oxidised LDL levels (-6.65 ± 16.14 ng/ml, $p= 0.042$). Results are compatible with most of the studies in the literature which showed that PRDC exerts a lowering effect on LDL oxidation (Osakabe et al 2001; Wan et al 2001; Baba et al 2007a; Baba et al 2007b). The beneficial effect of PRDC on oxidised LDL levels along with its effect on IR might not be attributed to a change in physical activity or in the usual diet of participants, as the latter lifestyle factors did not significantly change throughout the intervention (as reported by participants).

The decrease in LDL oxidation levels in this study is particularly important, as it suggests a potential role of PRDC (500 mg of polyphenols) in lowering oxidative stress, and consequently, in decreasing risk factors for many diseases such as CVD, hypertension and diabetes (Urquiaga and Leighton 2000).

The mechanism possibly involved the scavenging ability of polyphenols (Baba et al. 2007b), and their binding to LDL particles, resulting in their protection from oxidation (Haslam 1981). Wan et al. (2001) explained that the long term exposure of LDL particles to flavonoids (e.g. 4 weeks) renders LDL less susceptible to oxidation (Wan et al. 2001). The effect of PRDC on LDL oxidation was previously suggested to be due to an increase in HDL which suppresses oxidation (Wan et al. 2001; Mursu et al. 2004) through the inhibitory effect of paraoxonase on PAF-AH oxidation. The latter increases the degradation of oxidised lipids in the arteries (Mertens and Holvoet 2001). However, there was no significant change in HDL levels in the PRDC group. HDL levels, albeit increased, remained non significant (0.08 ± 0.27 mmol/l, p= 0.12) in contrast to the studies of Mursu et al. (2004) and Wan et al. (2001). It was postulated that HDL prevents the effect of LDL on monocyte infiltration (which is responsible of the development of fatty streak), and leads to a decrease in oxidised LDL levels (Mertens and Holvoet 2001). Lastly, the implication of NO in the inhibition of LDL oxidation (Cooper et al. 2008) cannot be excluded.

Studies that documented a lowering effect on LDL oxidation lasted 2 – 12 weeks, and used doses of flavonoids ranging from 174 - 651 mg (Osakabe et al 2001; Wan et al 2001; Baba et al 2007a; Baba et al 2007b). Hence, testing the impact of a lower dose of polyphenols (than 500 mg) on LDL oxidation in a longer term study might be helpful in making future recommendations using a lesser quantity of DC.

*Hs-CRP levels*

The current study did not show a significant effect of PRDC on hs-CRP levels. While epidemiological studies showed a positive correlation between this marker and CVD (Gisueppe et al. 2008), findings match with most of the intervention studies on dark chocolate, which showed no significant effects on hs-CRP levels (Mathur et al. 2002; Allen et al. 2008; Nogueira et al. 2012; Di Renzo et al. 2013). However, findings do not correspond with the studies of Hamed et al. (2008) and Stote et al. (2012), which reported a decrease in hs-CRP following cocoa/DC supplementation. Notably, the two latter studies were short term studies (5 days and

7 days, respectively), highlighting the possibility of an adaptation effect to the quantity of polyphenols in longer term studies. Also, since an epidemiological study showed that hs-CRP levels are lower in subjects consuming 1 serving of dark chocolate (20g) every 3 days compared to higher consumers ($p< 0.0001$) (Di Giuseppe et al. 2008), it can be hypothesized that a moderate consumption of chocolate might exert a lowering effect on inflammatory markers. This view is reinforced by the fact that most of the intervention studies involved daily consumption of chocolate (quantities ranging from 37g -100g with doses of polyphenols between 500 – 2135 mg), and showed no significant effects on hs-CRP levels. However, epidemiological studies do not analyse cause and effect, and can be confounded by many factors (CDC 2004) that are not related to chocolate. Therefore, testing the moderate consumption of DC on hs-CRP levels at various occasions (every week) is needed to elucidate the impact of PRDC on inflammation.

As a link between oxidative stress and inflammation via a NO-related mechanism was previously discussed (Cooper et al. 2008), it can be suggested that a decrease in LDL oxidation susceptibility might be associated with a decrease in inflammation. The fact that hs-CRP did not significantly change might have several explanations. The mechanism underlying the beneficial effect on oxidised LDL might not have involved NO (such as an increase in antioxidant enzymes) (Rahman et al. 2006). However, since NO levels or NOS enzymatic activity were not analysed in this study, no definite conclusions could be made. Also, the short-half life and the rapid elimination of flavonoids from the plasma were suggested as the possible reason of the non significant effect of these polyphenols on many inflammatory markers including IL-1, IL-6, TNF-α and hs-CRP (Mathur et al. 2002).
Nevertheless, one of the most plausible explanations to this result could be the reliability of hs-CRP test in detecting an increase or a decrease in inflammation following an intervention. In fact, the intra individual and inter individual variations related to this assay (Mccormack and Allan 2010) might have affected its accuracy. This suggests that although a strong predictor of coronary artery diseases (Ridker 2001), hs-CRP could not provide reliable results when measured on a single

occasion. In this study, the fact that this test was only analysed at baseline and after 4 weeks might have affected the identification of changes in hs-CRP over time. Further studies testing hs-CRP levels at different time points, while controlling for confounding factors (inflammatory conditions, acute infection etc…) might need to be considered before refuting a positive effect of PRDC on inflammation.

*BMI and waist circumference*

In the present intervention, no significant changes in BMI or WC following the consumption of PRDC were noted. Results matched with most of the intervention studies on PRDC lasting from 2-8 weeks, which assessed anthropometry as a part of a routine measurement (Engler et al 2004; Grassi et al. 2005; Kurlansdsky and Stote 2006; Allen et al 2008; Ried et al 2009; Al Moosawi et al 2010; Njike et al 2011; Al Moosawi et al 2012; Nogueira et al 2012). In this study, changes in body weight did not occur as a result of a decrease in insulin resistance, but corresponded with the fact that no changes in the reported energy intake and physical activity were noted ($p > 0.05$).

However, the increase in BMI only in the placebo group (as will be discussed later) but not in the PRDC group might suggest a possible counteracting effect of PRDC on the adverse effects of the fat and energy content of chocolate. This was previously suggested by Al Moosawi et al. (2012), which also noted an increase in BMI only in the placebo group following 4 weeks of daily consumption. In line with this, it was proposed that cocoa polyphenols might counteract the prooxidant effects of cocoa butter, and thus could help against an increase in weight as well an increase in insulin and glucose levels (Vinson et al. 2006). Results suggest the possibility of an inhibitory effect of cocoa polyphenols on lipid and carbohydrate absorption through the inhibition of digestive enzymes (Gu et al. 2011). This might have counteracted the increase in body weight in the PRDC group.

The outcomes could possibly match with the findings of animal studies which showed that cocoa polyphenols reduced weight gain in animals when administered high fat diets (Matsui et al 2005; Min et al 2011; Yamashita et al 2012). Although the aim of Study I was to include the placebo DC/PRDC in the context of a normocaloric diet, the increase in BMI might suggest that this product was

consumed in addition to the usual diet of participants. The countering effect of cocoa polyphenols on the fat content of chocolate might have prevented an increase in weight in the PRDC group.
The mechanism involved in the countering effect of PRDC on weight gain might be explained by the inhibitory effect of PRDC on fatty acid gene expression (Matsui et al. 2005) or on digestive enzymes (Dorenkott et al. 2014). As the possible mechanisms were only studied in animals, identifying these mechanisms in humans are then needed.

WC did not significantly change following PRDC consumption. Although a recent study showed that one week of daily PRDC supplementation (100 g) induced a decrease in waist circumference (($-1.24 \pm 1.45$ cm, $p \leq 0.05$) with no significant change in BMI (Di Renzo et al. 2013), this study is limited by many factors. In fact, the short term study, the small number of participants (15 participants) and the lack of a control group did not help making strong conclusions, and raise questions about the biological significance of this outcome.
Due to the limited studies on PRDC and its effect on body weight/body fat/obesity, more research well controlled for energy intake and physical activity is needed. In addition, the identification of the potential dose and fraction of flavonoids supposed to induce a counteracting effect on weight gain need to be studied.

*Adverse outcomes of placebo DC*

On the other hand, placebo DC was shown to increase insulin levels, insulin resistance (demonstrated by a decrease in HOMA-IR and an increase in QUICKI), glucose, TG, and BMI levels in adults. These parameters were recognized as being risk factors for chronic diseases (Grundy et al. 2004). The increase in HOMA-IR ($0.66 \pm 1.26$, $p=0.03$) and weight ($0.57 \pm 1.08$ kg, $p=0.011$) following 4 weeks of placebo DC consumption was only reported by the study of Al Moosawi et al. (2012) in which the placebo DC was similar to Study I. However, this only occurred in the overweight group. Although non significant, the increase in insulin levels ($p=0.07$) and HOMA-IR ($p=0.08$) following 8 weeks of low-polyphenol chocolate consumption (<2 mg of epicatechin) was also noticed by Mellor et al. (2010). In

relation to lipid profile, the detrimental effect of low-polyphenol chocolate was reported by the study of Wang-polagruto et al. (2006), which showed that consuming a low flavanol cocoa drink (43 mg of flavanols) resulted in a decrease in HDL by 9.6%, while an increase in HDL by 6.6 % was noted in the high flavanol cocoa drink (446 mg of flavanols). Also, Baba et al. (2007b) reported a beneficial effect of cocoa polyphenols on LDL oxidation, showed by a 9.4% increase in lag time after 12 weeks of flavanol-rich cocoa consumption ($p<0.001$). However, a significant decrease (19.8%) in lag time for LDL oxidation was noted in participants in the control group (consuming 12g of sugar).

The simultaneous change in insulin, HOMA-IR, QUICKI, glucose, TG and BMI levels might suggest a correlation between the markers. The increase in body fat was reported to increase IR, glucose and TG levels, as described by Kissebah et al. (1982). Indeed, the increase of fat and energy in the diet, might lead to an increase in free fatty acids. FFA could increase TG levels and possibly enhance hepatic glucose production (Brøns et al 2009; Jin et al. 2013) through an increase in total glucose output and the activity of glucose-6-phosphatase (stimulated by fatty acyl-CoA) (Lam et al. 2003). This might result in a greater insulin secretion, and a resultant increase in IR (Brøns et al. 2009). However, whether the increase in energy intake and the subsequent increase in body weight in this study were sufficient to induce this mechanism remain unclear.

The outcomes noted following placebo DC supplementation were not accompanied by a change in cortisol metabolism, the latter being known to play an important role in the metabolic syndrome (Qi and Rodrigues 2007), and insulin resistance (Masuzaki et al. 2001). This contradicts the findings of the previous study of Al Moosawi et al. (2012), which noted an increase in salivary cortisol following 4 weeks of placebo DC supplementation, along with an increase in fasting insulin and HOMA-IR. Results could be attributed to the presence of confounding factors such as fat and sodium intake, which might have masked changes in cortisol levels. Limited studies previously analysed the effect of low-polyphenol chocolate/PRDC

on cortisol levels and 11 β-HSD1 activity. Hence, trials investigating this association are needed.

The increase in BMI in the placebo DC was not associated with a significant change in the reported levels of physical activity. In addition, the analysis of diet diaries did not show a significant increase in energy intake between baseline and week 3 in the placebo DC and the PRDC groups. This might be mostly justified by underreporting, which is common in research studies (Bothwell et al. 1999). Underreporting, along with overestimating physical activity, was previously documented by the study of Davison et al. (2008).
Therefore, it could be suggested that participants of this study did not adhere to the dietary recommendations provided, and consumed the chocolate portion in addition to their diet, instead of replacing it by another food. Yet, the polyphenol content in the PRDC was able to counter the harmful effects of energy and fat in DC, while the placebo DC led to an increase in weight.

Results suggest that ordinary chocolate products in the market might have unfavourable effects on health. In addition, the outcome on TG levels refutes the theory that chocolate consumption has at worst a neutral effect on lipid profile, and suggests that more attention should be accorded to chocolate deficient in polyphenols. These detrimental effects of placebo DC are novel and suggest that studies targeting the effect of low-polyphenol chocolate on anthropometric and biochemical markers are needed. Also, because of the chocolate processing methods that cause a considerable loss of polyphenols (McShea et al. 2008), the analysis of polyphenol content of DC brands in the market might be needed to identify the ones that are deficient in polyphenols.

*BMI strata*

The current study did not note a significant impact of BMI on the outcomes when assessed by Mann whitney test, except for HOMA-IR in the placebo group. As expected, overweight individuals showed higher levels of SBP (p= 0.021), DBP (p= 0.034), insulin (p= 0.013), HOMA (p= 0,012), TC (p= 0.009), LDL (p= 0.002), HDL

(p= 0.003), TG (p= 0.003) and hs-CRP (p= 0.003) levels than normal weight individuals, and consequently a higher risk of metabolic complications (Silver et al. 2007). It was postulated that a higher BMI is associated with a greater insulin resistance, due to the fact that excess fat in skeletal muscle disrupts the insulin signalling pathway, leading to hyperinsulinemia and increased blood pressure levels (Addison et al. 2008). However, cortisol levels did not differ between normal weight and overweight individuals. It was previously shown that obesity causes an increase in the activity of HPA and cortisol levels, which eventually leads to an increase in insulin and TG levels (Ottoson et al. 1994). Also, visceral adipocytes have been reported to have more glucocorticoid receptors compared to subcutaneous adipocytes (Stulnig and Waldhausl 2004). Additionally, despite the fact that overweight and obese individuals are known to have a higher endothelial oxidative stress than normal weight people (Silver et al. 2007), there was no difference in the baseline levels of oxidised LDL between normal weight and overweight participants (p= 0.41). These findings might be attributed to the young study population or the small sample size of overweight individuals. However, no definite conclusions can be made regarding this finding.

This study showed that even with the differences in baseline levels in many parameters between the 2 BMI categories, and with the exception of HOMA-IR in the placebo group, overweight individuals did not respond differently to PRDC hypoinsulinemic and hypotensive properties, as previously suggested by Al Moosawi et al. (2012). A finding of this subgroup analysis corresponds to the meta-analysis of Ried et al. (2012) on blood pressure, which did not show an impact of BMI on SBP and DBP following cocoa/Dc supplementation. In the placebo DC group, the fact that the increase in BMI was not different between the normal weight and overweight population (p= 0.36) suggests that DC low in polyphenols might increase the risk of weight gain in normal weight individuals, and worsen obesity in the overweight population.

The fact that HOMA-IR was only increased in the overweight group (p= 0.017), while it was not affected in the normal weight group following placebo DC consumption is in line with the study of Al Moosawi et al. (2012), which used a

similar placebo DC product, and suggested that overweight individuals are more affected by the detrimental effects of fat and energy in the diet (Al Moosawi et al. 2012). However, the difference in response to HOMA-IR between the 2 BMI categories is intriguing since the increase in BMI, glucose and TG levels, and the decrease in QUICKI occurred in both groups with no significant impact of BMI. Despite the importance of these results, findings based on BMI subgroup analysis are limited by several factors such as the uneven number of participants between the normal weight and overweight group, and the small sample size when stratifying the population according to BMI and type of intervention. In addition, the adoption of non parametric tests presents disadvantages over the parametric tests, due to their lack of power and their lower accuracy compared to parametric tests (Whitley and Ball 2002). Therefore, the results might not help making strong conclusions. Studies that assess differences in responses to intervention based on BMI are needed to clarify this association.

*Qualitative data*

Data regarding acceptability reported adverse effects such as headache, gastrointestinal discomfort and unacceptability of chocolate consumption, mainly in the PRDC group. Unacceptability of treatment and complaints such as headache and constipation were also observed in other studies, mostly in the intervention group (5% of participants) compared to the control group (1% of participants) (Ried et al. 2012). This suggests that polyphenols and particularly flavanols might be responsible for most of these adverse effects, along with decreasing chocolate palatability due to their bitterness (McShea et al. 2008). The gastrointestinal discomfort was reported following the consumption of green tea rich in polyphenols (Chow et al. 2003). It was also proposed that doses higher than 500 mg of polyphenols were likely to cause side effects such as nausea and stomach upset (Mead 2007). In view of the potential role of polyphenols in decreasing carbohydrate and lipid absorption in the gut (Gu et al. 2011), the adverse effects noted in this study might be attributed to changes in bowel function mainly caused by the fraction of undigested substrates in the colon, particularly carbohydrates. The fermentation of undigested carbohydrates by intestinal bacteria in the colon leads to the production

of SCFA and gases (carbon dioxide, hydrogen and methane) which can cause flatulence and abdominal pain (Zopf et al. 2009). This may suggest that the effects of PRDC on decreasing fasting insulin levels and countering an increase in body weight, triglycerides and glucose levels were mainly due to the inhibitory effect of polyphenols on digestive enzymes.

The headache might be attributed the fact that some people lack sufficient amounts of the enzyme responsible for phenylethylamine (amine present in chocolate) breakdown (Beckett 2008). Additionally, the fact that chocolate was reported to trigger migraine might be due to the release of serotonin following chocolate consumption. Serotonin triggers a reaction that constricts blood brain vessels, resulting in a headache that is typical of migraine (Emsley and Fell 1999). Although headache was also attributed to stress that might precede cravings (Beckett 2008), this did not apply to the current study, since chocolate was asked to be consumed daily as a part of the intervention. Notably, headache and migraine are caused by chocolate consumption regardless of polyphenols.

In conclusion, this study showed a significant increase in insulin sensitivity and a decrease in oxidised LDL levels following PRDC consumption, while no effect on other parameters was noted. The adverse effects of placebo DC on anthropometric markers (increase of BMI) and biochemical markers (increase of TG, insulin, HOMA-IR and glucose and decrease of QUICKI) render the analysis of the polyphenol content of chocolate in the market necessary before establishing any recommendations.

## 4.2 Study II

### 4.2.1 Results

#### 4.2.1.1 Baseline and population characteristics

Fourteen volunteers completed the trial, and were mainly recruited from community centres in Mussselburgh. Ten participants were European, while the rest was sub-Saharan African (two participants), Eastern Mediterranean (one participant) and one

participant was described as having other ethnicities. Half of this population was employed while the others were students. Only 3 participants of this study were males. Five participants were obese (BMI between 30-34.9 $Kg/m^2$), and the rest were overweight. All volunteers had high levels of WC (classification of WC is presented in Figure 2.4), and were classified at high risk of metabolic complications, with the exception of one male participant who had a WC= 87cm. The baseline characteristics of the overall study population and split according to the type of intervention are presented in table 4.11.

**Table 4.11: Baseline characteristics of the study population**

| | Study population N=14 | Placebo group N=7 | PRDC group N=7 | Difference between groups Significance (p=) |
|---|---|---|---|---|
| | Mean ± SD | | | |
| Age (years) | 40.07 ± 13.3 | 35.29 ± 14.43 | 44.86. ± 11.04 | *0.2* |
| BMI ($Kg/m^2$) | 29.3 ± 3.45 | 29.31 ± 2.73 | 29.29 ± 4.29 | *0.1* |
| WC (cm) | 95.18 ±11.64 | 95.59 ± 11.96 | 94.76 ± 12.25 | *0.9* |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, WC: Waist circumference. Results are expressed as means ± SD. There were no significant differences between groups. Data was analysed using independent t-test.*

Regarding supplement intake, only one participant noted having a usual intake of dietary supplements in the form of multi-vitamins. Furthermore, 8 volunteers reported having a family history of chronic diseases such as diabetes, hypertension and/or CVD, and two females mentioned taking daily contraceptives. One woman was postmenopausal.

In relation to physical activity, 50% of the population reported practising more than 3 hours of exercise per week, and no significant differences in the baseline levels of physical activity (expressed by MET/ week) were noted between the placebo DC and the PRDC group (placebo DC: 8.8 ± 10.2 MET/ week; PRDC: 15.97 ± 12.46 MET/ week, $p = 0.26$).

#### 4.2.1.2 Anthropometric measures

Weight, BMI, waist circumference, lean body mass and body fat percentage levels were normally distributed for all interventions at all time points, as assessed by Shapiro-Wilk's test ($p > 0.05$). In addition, there was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). Moreover, none of the 3 above mentioned parameters violated the assumption of sphericity (Mauchly's test > 0.05).

*a. BMI levels*

There was no statistically significant interaction between the intervention and time on BMI levels ($F$ (2, 24) = 1.216, $p=0.31$). However, the main effect of time showed a statistically significant difference in BMI levels at the different time points ($F$ (2, 24) = 4.925, $p= 0.016$). Therefore, in order to determine where these differences lie, pairwise comparisons using Bonferroni test were carried out. For the overall group, changes in BMI levels were not statistically significant between pre intervention and mid way point (Mean difference = 0.12 $Kg/m^2$ (0.3, 0.12 CI), $p= 0.58$), but BMI levels increased significantly at post intervention compared to pre intervention (Mean difference= 0.41 $Kg/m^2$ (0.82, 0.007 CI), $p= 0.046$) (Table 4.12). Regarding between-group differences within mixed model ANOVA, the main effect of group showed that there was no statistically significant difference in BMI levels between intervention groups ($F$(1, 12) = 0.018, $p = 0.9$). This increase from baseline by 1.26 ± 1.76 Kg (BMI increased by 0.41 ± 0.57 $Kg/m^2$) occurred in the whole study population after 12 weeks.

**Table 4.12: Changes in BMI over time in the overall study population and split according to the type of intervention**

| | Means ± SD | | |
|---|---|---|---|
| BMI levels ($Kg/m^2$) | Study population (N=14) | Placebo DC (N=7) | PRDC (N=7) |
| BMI baseline | 29.3 ± 3.45 | 29.31 ± 2.73 | 29.29 ± 4.29 |
| BMI mid intervention | 29.42 ± 3.36 | 29.56 ± 2.53 | 29.27 ± 4.24 |
| BMI post intervention | 29.71 ± 3.57* | 29.94 ± 2.73* | 29.49 ± 4.47* |

**Significant difference from baseline, $p < 0.05$. BMI increased with no significant differences between the placebo and the PRDC groups ($p=0.9$). DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Results are expressed as means ± SD.*

*b. Waist circumference*

Results showed no significant interaction between the intervention and time on WC levels ($F$ (2, 24) = 1.955, $p= 0.16$), suggesting no effect of the type of intervention on waist circumference. Levels of WC albeit increased over time in the whole study population, did not reach statistical significance (($F$ (2, 24) = 3.08), $p= 0.065$). Regarding between group differences, there was no statistically significant difference in WC levels between intervention groups ($F$ (1, 12) = 0.06, $p = 0.81$) (Table 4.13).

**Table 4.13: Changes in waist circumference over time in the study population and split according to the type of intervention**

| | Means ± SD | | |
|---|---|---|---|
| WC levels (cm) | Study population | Placebo DC | PRDC |
| WC Baseline | 95.18 ± 11.64 | 95.59 ± 11.96 | 94.76 ± 12.25 |
| WC mid intervention | 95.6 ± 11.65 | 96.25 ± 12.23 | 94.94 ± 11.98 |
| WC Post | 96.31 ± 11.68 | 97.6 ± 12.18 | 95.01 ± 11.98 |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, WC: waist circumference. Results are expressed as means ± SD*

*c. Body fat percentage*

Mixed model ANOVA showed no effect of time and intervention on body fat percentage levels (F (1, 12) = 1.33, p=*0.27*). The main effect of time also showed no statistically significant difference in body fat percentage levels at the different time points (F (1, 12) = 0.01, *p= 0.92),* and no significant differences between groups were noted (F (1, 12) = 1.12, *p =0.31*) (Figure 4.8).

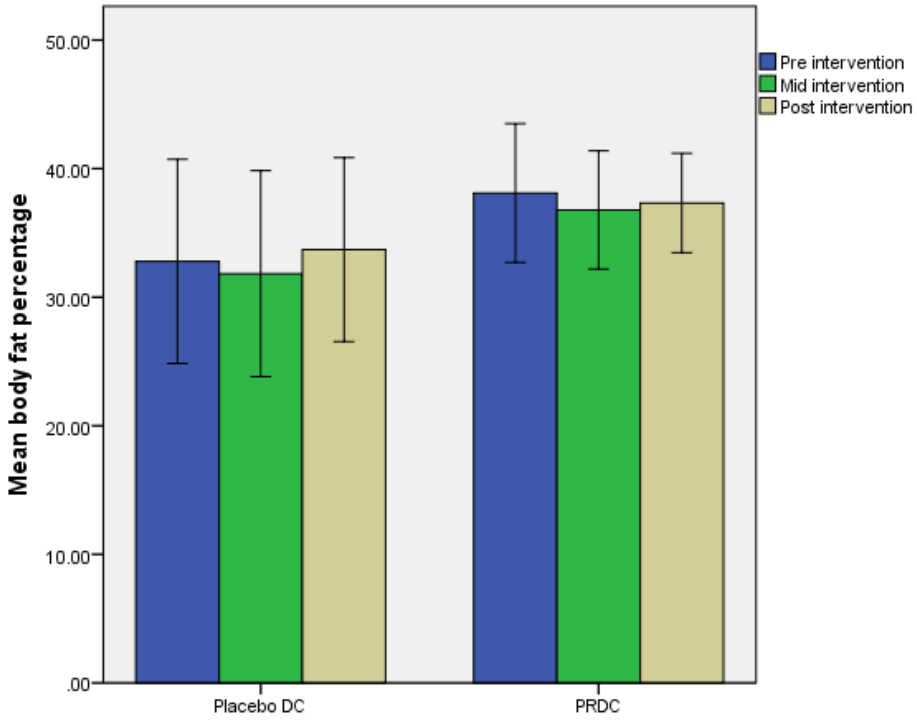



**Figure 4.8: Changes in body fat percentage in the placebo DC and PRDC groups throughout the intervention**

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Results are expressed as mean ± SEM. Bar charts represent the changes in body fat percentage at the 3 time points in both groups. Changes were not significant (p> 0.05). Baseline levels of body fat did not significantly differ between PRDC and placebo DC groups (p= 0.29).*

*d. Lean body mass*

Results documented no significant interaction between intervention and time on LBM (F (2, 24) = 0.071, *p= 0.93*). In addition, there was no significant effect of time on LBM levels (F (2, 24) = 1.1, *p= 0.93*), and no difference in this parameter

between the placebo and PRDC groups (F (2, 24) = 0.83, p= *0.38*) (Values of pre, mid and post LBM are presented in Table 4.16)

As analysis of results showed a significant increase in BMI without inducing changes in body fat percentage or LBM, repeated measures analysis for total body water were carried out to identify if changes in weight are due to fluctuations of body water. Results showed no significant changes in total body water resulting from the interaction between time and intervention (F (2, 24) = 0.15, p= *0.86*), or from the effect of time (F (2, 24) = 3.53, p = *0.54*).

#### 4.2.1.3 Basic metabolic rate

BMR levels were normally distributed for all interventions at all time points, as assessed by Shapiro-Wilk's test ($p > 0.05$). The assumption of sphericity was violated, as assessed by Mauchly's test of sphericity (*p=0.045*). Therefore, a Greenhouse-Geisser correction was applied (epsilon ($\varepsilon$) = 0.75). Results showed no statistically significant interaction between the intervention and time on BMR levels ($F$ (1.41, 16.9) = 0.21, *p= 0.74*). In addition, the main effect of time showed no significant difference in BMR levels at the different time points ($F$ (1.41, 16.9) = 0.13, *p= 0.8).* There was a slight difference in BMR levels between the two groups, albeit it did not reach statistical significance ($F$ (1, 12) = 4.31, *p =0.06* (Values of pre, mid and post BMR are presented in Table 4.16)

#### 4.2.1.4 Urine assessment

All participants provided urine samples pre, mid and post intervention. Total polyphenols, total antioxidant capacity and glucocorticoid levels were then analysed.

a. Cortisol, cortisone levels and the activity of the enzyme 11-β HSD

The analysis of glucocorticoids via ELISA method was previously validated for accuracy and precision in biological samples (Al Dujaili et al. 2012).
Cortisol, cortisone levels and 11β HDS1 levels were normally distributed for all interventions at all time points, as assessed by Shapiro-Wilk's test ($p > 0.05$). In

addition, there was homogeneity of variances, as assessed by Levene's test for homogeneity of variance ($p$ >0 .05). A violation of the assumption of sphericity (Mauchly's test > 0.05) was only noted for cortisone levels.

Cortisol levels were not significantly affected by the intervention and time ($F$ (2, 24) = 0.53, p=*0.59),* and there was a significant effect of time on these levels ($F$ (2, 24) = 2.61, p= *0.094).* Differences between groups were also not significant ($F$ (1, 12) = 2.55, $p$ = *0.14*).

For cortisone levels, a Greenhouse-Geisser correction was applied (epsilon (ε) = 0.701). Results showed no statistically significant interaction between the intervention and time on cortisone levels ($F$ (1.4, 16.81) = 0.71, p = *0.46).* There was also no effect of time on cortisone levels (F (1.4, 16.81) = 2.85, p = *0.1*), and no differences between the 2 groups ((F (1, 12) = 2.11, p = 0.17) regarding this parameter.

As for the activity of the enzyme 11 β-HSD 1, there was no statistically significant interaction between the intervention and time on 11 β-HSD 1 levels ($F$ (2, 24) = 1.076, p=*0.32).* Similarly, no significant effect of time was noted on 11 β-HSD 1 activity ($F$ (2, 24) = 0.55, p= *0.6).* Lastly, differences between the two groups were not significant ($F$ (1, 12) = 0.95, $p$ = 0.35) (Table 4.14).

*b. Total polyphenols and total antioxidant capacity in the urine*

Total polyphenols and total antioxidant capacity were obtained by comparing the absorbance against the generated standard curves prepared with gallic acid / ferrous sulphate standards, respectively. The standard curves are presented in Appendix 12.

Total polyphenols and antioxidant capacity were normally distributed for all interventions at all time points, as assessed by Shapiro-Wilk's (p>0.05). There was homogeneity of variances, as assessed by Levene's test ($p$ > 0.05), and none of the above mentioned parameters violated the assumption of sphericity (Mauchly's test > 0.05).

Results showed no significant interaction between the intervention and time on antioxidant capacity ($F$ (2, 24) = 0.97, $p=0.4$), and no main effect of time on these levels at the different time points ($F$ (2, 24) = 0.24, $p= 0.79$). Regarding between-group differences, the main effect of group showed that there was no statistically significant difference in urinary antioxidant capacity between intervention groups ($F$ (1, 12) = 3.25, $p = 0.097$).

As for the levels of polyphenols in the urine, there was no statistically significant interaction between the intervention and time on these levels ($F$ (2, 24) = 1.23, p=0.093), and no significant effect of time on polyphenol levels was noted (F (2, 24) = 0.36, $p = 0.7$). As for between-group differences, the main effect of group showed a statistically significant difference between the two groups ($F$ (1, 12) = 19.77, $p = 0.01$) (Table 4.14).

**Table 4.14: Changes in urinary glucocorticoids, antioxidant capacity and total polyphenols in the placebo and PRDC groups**

| | Intervention | Baseline | Week 6 | Week 12 |
|---|---|---|---|---|
| Cortisol (nmol/day) | Placebo DC | 67.97 ± 36.9 | 80.41 ± 47.97 | 97.5 ± 47.37 |
| | PRDC | 99.8 ± 44.75 | 137.7 ± 69.74 | 109 ± 64.73 |
| Cortisone (nmol/day) | Placebo DC | 97.52 ± 45.3 | 113.7 ± 44.6 | 136.7 ± 44.6 |
| | PRDC | 129 ± 56 | 190.9 ± 129.7 | 178.29 ± 94.64 |
| 11 β-HSD1 activity (cortisol/cortisone) | Placebo DC | 0.67 ± 0.16 | 0.7 ± 0.23 | 0.72 ± 0.2 |
| | PRDC | 0.8 ± 0.14 | 0.8 ± 0.25 | 0.74 ± 0.18 |
| Total antioxidant capacity (mM $Fe^{2+}$/day) | Placebo DC | 4.88 ± 1.4 | 6.62 ± 2.57 | 6.09 ± 1.96 |
| | PRDC | 8.32 ± 5.16 | 7.6 ± 1.06 | 8.22 ± 3.55 |
| Total polyphenols (mg of GAE/day) | Placebo DC | 69.92 ± 31.42* | 70.88 ± 24.8* | 99.12 ± 49.8* |
| | PRDC | 121.85 ± 67.92 | 168.94 ± 124 | 105.03 ± 54.86 |

**Significant difference between the placebo DC and the PRDC groups (p= 0.01). DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, GAE: Gallic acid equivalent, 11 β-HSD1: 11-beta hydroxysteroid dehydrogenase 1. Results are expressed as means ± SD.*

#### 4.2.1.5 Assessment of diet diaries and compliance

None of the macronutrient and energy values violated the assumption of normality (Shapiro-Wilk's ($p>0.05$)), homogeneity (Levene's test > 0.05) or sphericity (Mauchly's test of sphericity ($p> 0.05$). Reported energy intake did not change throughout the intervention in the placebo and PRDC groups ($F (2, 24) = 0.79$, $p = 0.47$). Regarding macronutrients, no significant changes in carbohydrate ($F (2, 24) = 0.62$, $p= 0.55$), or protein intake ($F (2, 24) = 1.34$, $p= 0.28$) were noted in both groups. However, there was a significant effect of time on fat intake levels ($F (2, 24) = 4.4$, $p = 0.024$). Yet, pairwise comparisons did not reveal significant differences in the levels of fat intake at different time points ($p> 0.05$). Finally, there were no differences between the 2 groups regarding fat intake ($p= 0.6$) as well as energy ($p= 0.5$), carbohydrate ($p= 0.26$) and protein ($p= 0.76$) intakes (Table 4.15).

**Table 4.15: Changes in energy and macronutrient intakes throughout the intervention in the placebo and PRDC groups**

| | Type of intervention | Run-in period | Week 6 | Week 12 |
|---|---|---|---|---|
| Energy (Kcal) | *Placebo DC (N=7)* | 1826 ± 401 | 1957 ± 760 | 1691 ± 439 |
| | *PRDC (N=7)* | 1764 ± 482 | 2070 ± 357 | 2005 ± 406 |
| Carbohydrate (g) | *Placebo DC (N=7)* | 251 ± 59 | 261 ± 86 | 233 ± 73.08 |
| | *PRDC (N=7)* | 188 ± 55 | 240 ± 98 | 199 ± 67 |
| Protein (g) | *Placebo DC (N=7)* | 58.06 ± 22.6 | 76.57 ± 9.99 | 74.7 ± 13.21 |
| | *PRDC (N=7)* | 70.76 ± 15.23 | 76.6 ± 34.22 | 70.9 ± 23.48 |
| Fat (g) | *Placebo DC (N=7)* | 72.4 ± 19.46 | 83.76 ± 16.87* | 87.93 ± 22.32* |
| | *PRDC (N=7)* | 81.04 ± 28.7 | 77.86 ± 33.04* | 66.43 ± 22.89* |

** Significant effect of time on fat intake in the placebo and PRDC groups, p=0.024. DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Results are expressed as mean ± SD*

Reported levels of physical activity (transformed into MET / hour) did not significantly change throughout the intervention in both groups ($F (2, 24) = 1.2$, $p= 0.32$) (Values for pre, mid and post MET are presented in table 4.16).

#### 4.2.1.6 Food Frequency questionnaire

Assessing changes in flavonoid intake through FFQ score showed no significant change in flavonoid intake in both groups between baseline, week 6 and week 12 ($F(2, 24) = 2.26$, $p = 0.13$). In addition, no between group differences ($F(1,12) = 4.2$, $p=0.53$) were noted (Baseline, mid and post values for FFQ are presented in table 4.16).

#### 4.2.1.7 Follow up appointment

Mixed model ANOVA showed no significant difference between the follow-up BMI value and the BMI of each of the 3 appointments, resulting from the interaction between treatment and time ($F(3, 33) = 0.91$, $p=0.45$) or from the effect of time ($F(3, 33) = 0.36$, $p=0.086$). Results suggest that changes in BMI were not significant after four weeks of the end of the study.

Regarding other parameters, there were no significant differences between the 3 appointments and the follow-up appointment for waist circumference (p=0.34), body fat percentage (p=0.7) and LBM (p=0.32) in both groups. In addition, the reported intake of flavonoids did not seem to significantly change after the end of the intervention when assessed by FFQ score (p=0.76). Reported levels of physical activity assessed by MET/hour did not also change significantly a month after the end of the study ($p= 0.49$). Similar results were noted for energy (p= 0.36) and macronutrient intake (carbohydrate (p= 0.39), protein (p= 0.63) and fat (p= 0.07)) in both groups at the follow up appointment. Lastly, there was no significant change in BMR (p= 0.57) at follow-up (Table 4.16).

**Table 4.16: Changes in assessed parameters from baseline to follow-up**

| | Intervention | Baseline | Week 6 | Week 12 | week 16 (Follow-up) |
|---|---|---|---|---|---|
| BMI ($Kg/m^2$) | Placebo DC<br>PRDC | 29.31 ± 2.73<br>29.29 ± 4.29 | 29.56 ± 2.53<br>29.27 ± 4.24 | 29.94 ± 2.73<br>29.49 ± 4.47 | 29.66 ± 2.9<br>29.6 ± 4.52 |
| WC (cm) | Placebo DC<br>PRDC | 95.59 ± 11.96<br>94.76 ± 12.25 | 96.25 ± 12.23<br>94.94 ± 11.98 | 97.6 ± 12.18<br>95.01 ± 11.98 | 96.82 ± 13.82<br>95.9 ± 12.37 |
| BMR (Kcal) | Placebo DC<br>PRDC | 1875± 353<br>1581 ± 90* | 1844 ± 358<br>1605 ± 95* | 1872 ± 248<br>1608 ± 102* | 1882±282<br>1586 ± 83* |
| Body fat percentage (%) | Placebo DC<br>PRDC | 32.79 ± 10.5<br>38.1 ± 7.14 | 31.8 ± 7.14<br>36.79 ± 6.08 | 33.7 ± 9.47<br>37.3 ± 5.12 | 31.52 ± 8.92<br>37.07 ± 5.88 |
| LBM (%) | Placebo DC<br>PRDC | 64.37 ± 6.34<br>61.87 ± 7.11 | 63.74 ± 7.31<br>60.45 ± 7.44 | 62.43 ± 7.98<br>58.84 ± 6.85 | 68.46 ± 8.97<br>62.7 ± 5.75 |
| Energy intake (Kcal) | Placebo DC<br>PRDC | 1826 ± 404<br>1764± 482 | 1957 ± 760<br>2070 ± 357 | 1691 ± 439<br>2005 ± 406 | 1786 ± 453<br>1793 ± 302 |
| Carbohydrate intake (g) | Placebo DC<br>PRDC | 262 ± 56<br>188 ± 55 | 265 ± 94<br>240 ± 98 | 230 ± 79<br>199 ± 67 | 213.8 ± 58<br>190 ± 58 |
| Protein intake (g) | Placebo DC<br>PRDC | 58.06 ± 22.6<br>70.76 ± 15.23 | 76.57 ± 9.99<br>76.6 ± 34.22 | 74.7 ± 13.21<br>70.9 ± 23.48 | 69 ± 25.97<br>70.02 ± 12.32 |
| Fat intake (g) | Placebo DC<br>PRDC | 72.4 ± 19.46<br>81.04 ± 28.7 | 83.76 ± 16.87<br>77.86 ± 33.04 | 87.93 ± 22.32<br>66.43 ± 22.89 | 76.53 ± 20.96<br>69.23 ± 18.1 |
| Flavonoid intake score (FFQ) | Placebo DC<br>PRDC | 361±133<br>443 ±292 | 295 ± 136<br>475±268 | 408±201<br>416±241 | 361±239<br>402±227 |
| MET/week | Placebo DC<br>PRDC | 10.3 ± 10.32<br>15.97 ± 12.46 | 10.77 ± 9.56<br>12.19±10.27 | 8.93 ± 7.73<br>14.04±12.49 | 9.43 ± 6.85<br>12.19 ± 8.03 |

** Significant difference between the placebo and PRDC groups for BMR (p=0.044). No other significant differences between groups were noted. DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate, WC: Waist circumference, BMR: Basic metabolic rate, LBM: Lean body mass, FFQ: Food frequency questionnaire, MET: Metabolic equivalent task.*

In order to identify the accuracy in reporting energy intake via diet dairies in this population, energy expenditure for each participant was calculated based on energy expenditure, which was estimated via BIA. Levels of physical activity were the ones reported by participants. The comparison between mean reported levels of energy

intake and calculated energy expenditure is illustrated in Figure 4.9. The ratio of calculated energy expenditure to reported energy intake ranged between 1.11 -1.25.

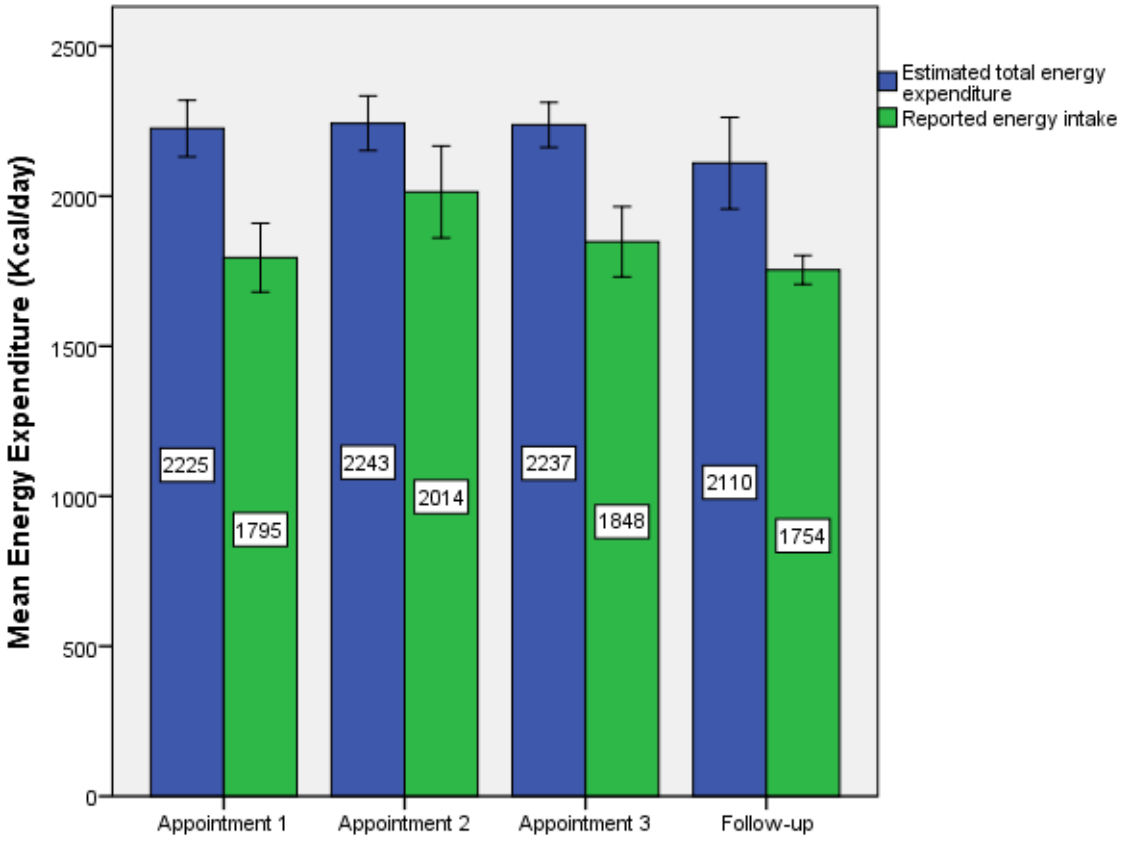



**Figure 4.9: Differences between mean estimated daily levels of energy expenditure and reported levels of energy intake in the study population**

4.2.1.8 Pearson's correlation between selected parameters

In the current population, BMI levels were significantly correlated to WC levels ($r = 0.85$, $p<0.001$), and to body fat percentage levels, but to a lesser extent ($r = 0.68$, $p=0.007$). However, changes in BMI at week 12 from baseline were not significantly correlated to changes in body fat percentage ($r = 0.43$, $p=0.13$), as well as waist circumference ($r=0.49$, $p=0.08$) between baseline and week 12.

#### 4.2.1.9 Eating patterns

The influence of chocolate consumption on the changes in eating patterns was mainly assessed through looking at sugar and saturated fatty acid intakes. Values were based on the self-reported diet diaries. For sucrose, results showed no significant change in these levels ($F (2, 24) = 0.029$, $p= 0.97$) throughout the intervention (Pre intervention: 34.37 ± 4.75 g; week 6: 32.01 ± 19.3 g; week 12: 28.5 ± 14.37 g ) in both groups. The analysis of subgroups of fat also showed no significant change in saturated fatty acids (SFA) ($F (2, 24) = 2.48$, $p= 0.12$) following the intervention in the placebo and PRDC groups (Pre intervention: 23.59 ± 9.7 g; week 6: 26.02 ± 10.56 g ; week 12: 25.71 ± 9.12 g). Also, no significant differences between the placebo DC and the PRDC groups were noted for sugar ($p=0.32$) and SFA intakes ($p=0.46$).

#### 4.2.1.10 Qualitative data: acceptability and side effects of placebo / treatment

Data regarding acceptability showed that none of the 14 participants found the chocolate unacceptable, while 9 participants found it very acceptable, and 5 participants found it somewhat acceptable. Few side effects were noted. In the PRDC group, complaints due to bitterness and headache (one participant), bloating and gastrointestinal discomfort (one participant) were reported. In the placebo DC group, one participant complained of the high caffeine content in the chocolate. Five participants in the placebo DC group and 5 in the PRDC group stated that they will continue consuming chocolate frequently. Regarding the change in appetite, 3 participants noted a change in appetite following DC consumption: One participant in the PRDC group and two participants in the placebo DC group noted a decrease in the consumption of sweet snacks due to daily chocolate consumption, yet this was not supported in the diet diaries of these participants. In addition, two participants reported an influence of recording diet diaries on improving healthy eating (eating less sweets and snacks) and increasing physical activity, regardless of the type of chocolate administered.

#### 4.2.1.11 Feasibility outcomes

*a. Drop out rates*

Six participants dropped out throughout the study. Five participants withdrew during the first 6 weeks, while one participant dropped at week 8. Reasons for dropout included: infection (1 participant), work commitment (1 participant), personal problems (2 participants). Two participants dropped out without giving any reasons. Therefore, the dropout rate was estimated to be 30 %.

*b. Compliance rates*

The compliance was estimated to be 84.82 %. This was calculated by counting the returned plastic bags, and asking participants (each 6 weeks) about the number of times they missed the daily portion of chocolate. The percentage of compliance was calculated for each participant, and the average percentage was considered for the fourteen participants. The efficiency of data collection tools and the study design are examined in section 5.2.2.

*c. Sample size calculation for a large trial*

The outcomes of the small scale study were used to estimate the sample size needed for a larger study. This was based on the estimates of means and standards deviations of the differences in BMI levels in the two independent groups at different time points. Determination of sample size was set at 80% power and a two-sided significance level of 0.05, and was based on the sample size tables in clinical studies by Machin et al. (1997) (CUHK 2014) (Table 4.17).

**Table 4.17: Estimation of sample size for a future large scale study**

| BMI SD differences | Mid – pre (Placebo DC) | Mid-pre (PRDC) | Post – pre (Placebo DC) | Post –pre (PRDC) | Post – mid ( Placebo DC) | Post- mid PRDC |
|---|---|---|---|---|---|---|
| | 0.117 | 0.335 | 0.414 | 0.57 | 0.296 | 0.597 |
| Sample size per group (total sample) | 130 | (260) | 31 | (62) | 65 | (130) |

*DC: Dark chocolate, PRDC: Polyphenol-rich dark chocolate. Calculations were based on 80% power for an alpha error of 0.05 using standard deviations (SD) of the mean differences at different time points in each group based on the tables by Machin et al. (1997).*

If the highest number is considered, it can be then assumed that a sample size of 260 participants is required for a power of 80 % and 0.05 level of significance. Considering the drop out rate of 30%, 338 participants are then needed to be recruited for a large trial.

In summary, the results of this study showed that 12 weeks of the consumption of DC low or rich in polyphenols led to an increase in BMI, while no changes in WC or body composition were noted. The intervention did not result in significant changes in urinary antioxidant capacity and polyphenol levels, as well as in glucocorticoid metabolism in both groups.

### 4.2.2 Discussion

The discussion part comprises two sections: The first section will discuss the outcomes of the study, while the second section will consider the feasibility outcomes and sample size calculations for a large trial.

#### 4.2.2.1 Section I: Discussion of the outcomes

This study generated preliminary results on the effects of PRDC supplementation on body weight and composition in the overweight and obese population. The inclusion of participants with an upper range limit for BMI (between 25 - 34.9 Kg/$m^2$) was considered because of the high complications associated with obesity class II and morbidly obese participants (Gibson 2005). In addition, there might be inaccuracy in measuring waist circumference for severely obese individuals due to the difficulty in finding the waist narrowing; thus, the measurement of WC is commonly taken at the umbilical level (Lohman et al. 1988). Also, it appears there is no validation for bioelectrical impedance for individuals with BMI over 34 Kg/$m^2$ (Kyle et al. 2004b; Coppini et al. 2005).

Based on the arguments exposed in section 2.2.1, there was an important rationale behind carrying out a human study testing the potential weight-lowering effect of PRDC. This is particularly useful for subsequently including this snack in weight management programs aiming to reduce or manage lifestyle-related diseases. However, rejecting this hypothesis might have a significant implication. In fact, there is currently a growing marketing that is promoting the benefits of DC in reducing body weight and counteracting obesity. This might be misleading if not supported or refuting by human intervention studies.

*BMI*

The increase in BMI (0.41 ± 0.57 Kg/$m^2$) occurred in the whole population after 12 weeks, with no significant difference between placebo DC or PRDC consumers ($p= 0.9$). The increase in BMI in the placebo group was previously documented by the study of Al Moosawi et al. (2012) following 4 weeks of daily consumption of 20g of DC (providing 102 Kcal). As for PRDC, an increase in body weight (0.8 Kg) previously occurred in 43 human participants following 3 months of daily consumption of 25g of flavanol-rich dark chocolate (containing 125 Kcal and providing 112 mg of flavanols) (Desch et al. 2010). However, whether this amount of flavanols is considered high was not previously discussed, but it is nearly four

times lower than the amount of flavanols contained in the PRDC (400mg) in Study II.

The increase in weight in this study population might be primarily explained by the fact that participants in both groups did not adhere to the dietary recommendations provided, and did not substitute the chocolate by another food. Therefore, DC was consumed in addition to the usual diet. Notably, the increase in BMI in the PRDC group did not reinforce the findings of "in vitro" studies which suggested a role of cocoa polyphenols in decreasing obesity due to their inhibitory actions on carbohydrate and fat digestion and absorption (Gu et al. 2011; 2013). Also, results did not support the outcomes of animal studies which showed that cocoa polyphenols were effective in attenuating weight gain in obesity-induced animals (Matsui et al. 2005; Yamashita et al. 2012; Gu et al. 2013).

However, despite the non significant differences between the placebo DC and the PRDC in relation to weight gain ($p= 0.9$), the increase in BMI in the placebo group was linear while the increase in the PRDC group mainly occurred after week 6 (Table 5.11). Also, considering the weight gained in each group, there was a greater increase in weight ($1.9 \pm 1.9$ Kg) in the placebo DC group after 12 weeks, compared to the PRDC group ($0.63 \pm 1.45$ Kg). This might suggest the probability of an adaptation effect to the quantity of polyphenols over time, which might limit their efficiency in counteracting weight again over the long term. In fact, it could be possible that PRDC was able to offset the increase in weight for the first 6 weeks, but not for the next 6 weeks of intervention. The idea of "adaptation effect" was previously suggested by Al Moosawi et al. (2012), and could provide an explanation for the efficacy of PRDC in counteracting weight again in a 4-week study (Al Moosawi et al. 2012). Yet, the small sample size did not help making strong conclusions regarding the implication of PRDC in attenuating body weight gain.

This increase in weight after 12 weeks is not only statistically significant, but can also be clinically relevant. It was shown that an increase of 1 Kg of body weight can result in an increase of coronary mortality risk by 1 - 1.5 % (Jousilahti et al. 1996). Therefore, a careful attention should be provided to the extra calories provided by the daily consumption of this snack.

An important point to mention is that previous animal studies assessed the counteracting effect of cocoa polyphenols on a high-fat induced diet. However, the implication of these polyphenols in reducing obesity in the context of a normocaloric or a hypocaloric diet remains unclear. For instance, Si et al. (2011) showed that administering cocoa-derived epicatechin to obese mice without a high fat diet, improved their biochemical markers without influencing body weight. This raises questions over the implication of these components in decreasing weight in humans; as if they are mainly involved in counteracting induced obesity, their efficacy in weight management programs or in reducing weight in individuals following a normocaloric diet becomes doubtful. Therefore, based on animal studies, it could be suggested that cocoa polyphenols play a positive role only when a high fat diet is induced, underlying a possible role of these polyphenols in countering weight gain in the population. However, data are inconclusive and further studies targeting animals and humans are needed. For instance, animal studies that include control group (no cocoa polyphenols and no fat induced diet), intervention group 1(administered cocoa polyphenols and high fat induced diet) and intervention group 2 (administered cocoa polyphenols along with a normal diet) might be helpful in investigating the mechanism of action of cocoa polyphenols on body weight.

Moreover, since the decrease in fat and glucose absorption through the inhibition of digestive enzymes was suggested as an important mechanism by which cocoa polyphenols might prevent obesity (as suggested by Gu et al. (2011)), the timing of intake of cocoa/DC (with meals or with no foods) and meal patterns might affect this factor. Nevertheless, in this study, there was no restriction on the timing of chocolate intake. While looking at the diet diaries in the PRDC group, four participants reported having the chocolate mostly at an afternoon snack with tea or coffee, while the others reported having it mainly with lunch and breakfast. Consequently, differences arising from the fact that no restriction on the timing of intake was made, might have affected the outcomes. Additionally, it was suggested that the macronutrient composition of a meal might influence polyphenol absorption, the latter being the highest after a carbohydrate-rich meal (Schramm et al. 2003). Therefore, a study well controlled for the timing of intake of PRDC is important to

surmount this limitation. To explore further, a UK cohort study including 1618 participants who reported their diet by the means of diet diaries, showed that lunch and dinner constitute the largest meals of the day with the highest proportion of carbohydrates (39.5 % of lunch and 36.6 % of dinner) (Al Moosawi et al. 2013). Consequently, asking participants to consume this snack with lunch or dinner in future studies, might help reducing or eliminating the confounding factor of consuming this snack with different meals or with no foods.
Notably, this inhibitory effect of cocoa polyphenols on digestive enzymes was only demonstrated in "in vitro studies" which cannot substitute for human studies (Etcheverry et al. 2012). In addition, the doses of polyphenols that exert an inhibitory effect in cells might be higher than the physiological levels. Hence, human studies investigating the effect of PRDC on macronutrient digestion and absorption, while controlling for the timing of chocolate intake are needed.

There is also a question about the dose of flavanols needed and the frequency of DC consumption required to induce a weight-lowering effect. PRDC (containing 500 mg of polyphenols and providing 102 Kcal) did not result in a weight reduction but in an increase in weight in this study. In addition, Gu et al. (2013) stated that the amount that reduced body weight in rats was equivalent to 513 mg of flavanols (provides 221.4 Kcal). Yet, more studies are needed to identify the amount supposed to decrease weight in humans. Furthermore, since procyanidins were suggested to decrease weight reducing effect compared to the monomeric flavanols (Dorenkott et al. 2014), it is important to identify the fraction of polyphenols that is most likely to induce a lowering effect on body weight.

Furthermore, although studies showed that the total calorie content of a diet counts more than fat intake in weight loss regimens (Harvey-Berino 1999; Djuric et al. 2002), the difference in fat content between cocoa and DC might have affected the ability of polyphenols in DC to reduce weight. It was stated in a review that the beneficial effects of cocoa, and particularly its weight-lowering properties might not be applicable to dark chocolate (Ali et al. 2014). In addition, cocoa was administered to animals in the context of a controlled diet. However, humans exhibit day-to-day

fluctuations in their daily food intake (Monsen and Van Horn 2008). This will affect the identification of the true effect of DC on weight. This is particularly difficult when looking at small changes in body weight in the context of a normo caloric diet. Further studies including a large number of participants, controlling for human diets, and providing details on the flavonoid and fat contents of chocolate should be considered to identify the effect of DC on body weight.

Lastly, it is worth mentioning that the term "anti-obesity" which was used in many reviews and studies (Matsui et al. 2005; Min et al. 2012; Ali et al. 2014; Dorenkott et al. 2014) might be overstated. In fact, obesity is a multifactorial condition, and it is very unlikely that one snack/food could help protecting against it. This term might lead to an overestimation of the ability of polyphenols in countering a small proportion of fat and energy in the diet. Thus, this designation might need to be reconsidered.

*Body composition*

The increase in BMI noted in this study was not associated with significant changes in body fat percentage, lean body mass or total body water, when the latter parameters were assessed by BIA. This was further documented by the non significant correlation between changes in body fat percentage and changes in body weight from baseline at week 12 (r= 0.43, $p= 0.13$). The increase in BMI cannot be attributed to a change in the amount of clothing since participants were asked to wear approximately the same clothes on each occasion. Although participants were asked to reduce the intake of caffeine and alcohol prior to the BIA test, the non significant changes in body composition might be related to the difference in the level of hydration of participants, which my affect the accuracy of BIA (Dehghan and Merchant 2008). In addition, even though women were asked about the day of the menstrual cycle during each appointment, results were unclear. Females did not show an increase in their weight when the appointment was in the last days of their cycle. Although a confounding factor, it might be difficult to control for the fluctuations in the levels of hydration related to the menstrual cycle of female participants, due to the fact that appointments are fixed at a nearly specific time.

*Waist circumference*

Despite the increase in BMI, no significant changes in waist circumference were noted. This also corresponded to the non significant correlation between changes in BMI and changes in WC from baseline throughout the study ($r= 0.49$, $p= 0.08$). The increase in weight cannot therefore be explained by an increase in muscle mass (since LBM did not significantly change, $p<0.05$), and might suggest that changes in weight were not sufficient to induce changes in WC. As WC is an important marker of diseases (Snijder et al. 2006), further studies are needed to investigate this relationship, particularly that PRDC was involved in decreasing WC. This was noted in the short-term study of Di Renzo et al. (2013), which showed a decrease in waist circumference ($–1.24 \pm 1.45$ cm, $p \leq 0.05$) after one week of PRDC consumption (with 2000 mg of polyphenols). However, due to the lack of a control group in the latter study, there might be an expectation bias which resulted in a decrease in food intake during the week of intervention. Also, because of the short study duration, results were inconclusive. Long term placebo-controlled studies are then required to identify any potential decrease in WC resulting from PRDC consumption.

*Reported energy intake through diet diaries*

The increase in BMI was not associated with an increase in energy intake as reported by the diet diaries. One of the most plausible explanations for these findings is underreporting. The inaccuracy of self-reporting in estimating the actual intake was previously noted, particularly in the obese population (Schoeller 1995; Goris et al. 2000), and BMI status was considered the major contributing factor to underreporting (Bram et al. 1998). It was demonstrated that in obese individuals, the amount of nitrogen excreted by the urine is equal to half the amount of nitrogen when calculated through the self-reported dietary intake. In another study, the reported energy intake constituted $59 \pm 24$ % of the actual energy expenditure in obese individuals when validated through doubly-labelled water. This discrepancy was proportional to body fatness. Underreporting was also described by Davison et al. (2008), who suggested an underestimation of energy intake and an overestimation of physical activity in overweight individuals when assessing the effect of cocoa polyphenols on blood pressure and other markers. In addition to BMI, the

underestimation was higher in females compared to males (Bandini et al. 1990). While the reasons of underreporting are not completely elucidated, inability to describe the diet (Schoeller 1995), undereating and underrecording (Goris et al. 2000) were proposed.

In this study, the ratio of calculated energy expenditure to reported energy intake ranged between 1.11 - 1.25. It was previously suggested that a ratio higher than 1 indicates underreporting (Garriguet 2008). However, this ratio of 1.11-1.25 represents the mean level of underreporting in the whole study population. By looking at the reported energy intake for each participant, 8 participants constantly underreported their energy intake, while 4 participants adequately reported and 2 participants overestimated their daily energy intake. It might be then more accurate to look at the diet diaries of each participant in order to identify underreporting. In addition, the application of a correction factor to the self-reported energy intake via diet diaries might be helpful in estimating the actual energy intake among the population.

ANOVA test showed a significant effect of time on fat intake throughout the intervention, as assessed by diet diaries ($p= 0.024$). However, pairwise comparisons did not show a significant change in fat intake at different time points ($p>0.05$). Fat intake seemed to be increased in the placebo DC group, and decreased in the PRDC, yet this did not reach statistical significance. Although it appears contradictory, getting significant ANOVA results with no significant difference in pairwise comparisons has been previously reported in the literature (Westfall 1999), and might be explained by the requirements for larger differences to declare significance in pairwise comparisons. Other explanations could be the adoption of a univariate approach which is considered less powerful than a multivariate output (such as Wilk's lambda and Pillai's trace) when the sample sizes are small (Stevens 2013). In the case of fat intake, pairwise comparisons seemed to be consistent with the non significant results of the multivariate tests (Wilk's Lambda for the interaction between time and intervention = 0.092). This discrepancy between multivariate and univariate tests is uncommon and has been attributed to many factors such as the presence of outliers (Park et al. 2009). It would then be more recommended to adopt

the results of multivariate tests since the latter do not need the sphericity to be assumed. Nevertheless, multivariate tests might also be limited by the small sample size, which reduces the numbers of degrees of freedom for error, and might therefore affect their accuracy (Park et al. 2009).
As a result, it could be suggested that there were no significant differences in fat intake following chocolate consumption in both groups, and that more studies with a larger sample size are needed to identify a potential effect of DC intervention on fat intake.

*Basic metabolic rate*

As for BMR, this study did not show an increase in energy expenditure in both groups. Results did not reinforce the findings of an animal study which reported an increase in energy expenditure in rats consuming cocoa flavanols (0.2 %) for 2 weeks (by 42 Kcal/ 22h, $p<0.05$) through the activation of mitochondrial biogenesis (Osakabe 2013). On the other hand, it is known that the increase in weight is generally related to an increase in energy expenditure (Schoeller 1998), which is responsible for decreasing the pace of weight again over the long term when extra calories are consumed. It could be possible that the increase in weight in this study was not sufficient to induce an increase in energy expenditure. However, the most plausible explanation for this outcome is the fact that energy expenditure was estimated via the BIA machine. This estimation is based on lean body mass rather than the measurement of actual oxygen consumption and carbon dioxide production, which is generally performed via indirect calorimetry (McClave and Snider 1992). Consequently, the non significant changes in lean body mass provided an explanation for the non significant change in BMR. Further studies well controlled for energy intake and physical activity, and using accurate methods for measuring energy expenditure (such as indirect calorimetry) are then required.

*Follow up appointment*

The follow-up appointment documented no significant changes in BMI levels. As shown, participants maintained the gained weight four weeks after the end of the intervention. Results seemed to be consistent with the non significant change in

energy and macronutrient intake recorded after the end of the study. While taking into consideration that the sample size does not help making strong conclusions, it might be possible that participants adapted to the extra snack consumed daily, and maintained the habit after the study. Notably, although significant differences between the placebo DC and PRDC groups were noted for BMR ($p=0.044$), this could be mainly explained by the difference in baseline levels of BMR between the groups. This finding is intriguing since there is no significant difference in LBM levels between the two groups ($p=0.54$). Yet, this did not affect the outcomes over time.

*Eating patterns*

One of the novel suggestions of study II was to assess a potential change in eating patterns through the analysis of the intake of sugars and saturated fats following DC consumption. Diet diaries did not show a significant difference in sugar intake at different time points. In addition to the possibility of a non significant effect of daily DC consumption on sugar intake, this result might be due to the difficulty in measuring sugar intake through self-reported data. In fact, a study assessing sugar intake using a biomarker of sugar in the urine and self-reported techniques (FFQ, diet diaries and 24-hour dietary recall) showed an inaccuracy of the self-reported techniques in estimating sugar intake (pearson's correlation between sugar intake and self reported techniques ranged between 0.22 and 0.27, $p > 0.05$), which was shown to be underreported. This stresses on the use of a biomarker of total sugar in the urine as a way to get a proper estimate of sugar intake (Tasevska et al. 2014). This point deserves more investigation, and looking at whether a consumption of a sweet snack can decrease the exposition to temptations and promote healthy eating (Kroese et al. 2009) need to be considered.

Given all the facts previously exposed, research on DC and its weight lowering properties remains novel and many studies targeting the suggested mechanisms of action are needed. These mechanisms include the effect of PRDC on the inhibition of digestive enzymes (Gu et al. 2013), and the increase in fat oxidation and gene fatty acid expression (Matsui et al. 2013); the modulating effect of PRDC on food intake

and satiety through the action of polyphenols on neuropeptides via the central nervous system (Panickar 2013); and the effect on thermogenesis (Osakabe 2013). Also, since the increase in insulin sensitivity due to polyphenols was proposed as one of the mechanisms that decrease obesity (Panickar 2013), studies that investigate the long term effects of PRDC on body weight while assessing glucose and insulin levels, are needed to possibly explain this effect. Nonetheless, it is crucial to mention that given future controlled studies might note a significant decrease in body weight following PRDC consumption, it is unlikely that these changes will be of clinical relevance. However, this will be useful in incorporating PRDC in hypocaloric diets aiming to help in weight loss.

*Biomarkers of compliance*

As for biomarkers of compliance, this study did not show a significant change in the levels of total antioxidant capacity and total polyphenols in the urine. The results contradict studies in the literature that showed an increase in urinary polyphenols following PRDC consumption (Ito et al. 2005; Al Moosawi et al. 2010; Al Moosawi et al. 2012). Even though compliance to DC consumption was reported to be high (84.82 %) in this study, levels of polyphenols and antioxidant capacity in the urine did not validate the results. These outcomes might be explained by the small sample size which did not help to identify the correlation between PRDC consumption and polyphenol levels and antioxidant capacity in the urine. Furthermore, outcomes might be explained by the fact that, with the exception of other cocoa and chocolate products, participants were not asked to limit the intake of foods rich in flavonoids. However, this might have masked the increase in phenolic levels in the urine following PRDC consumption. In addition, two participants provided huge differences in the volume of the collected 24-hour urine between the 3 samples, which might be due to their decrease in compliance, rather than a decrease in their urine output. This might have influenced the assessment of the change in antioxidant and polyphenol levels. Finally, the intra and inter individual variability in the metabolism of polyphenols cannot be excluded (Santos-Buelga et al. 2010).

However, although studies have discussed the importance of evaluating compliance in the urine (Pérez-Jiménez et al. 2010), there are many limitations related to this test. In fact, the assessment of polyphenols in the urine is limited by the short half-life of these components (1-5 days) (Santos-Buelga et al. 2010). Consequently, the ability of this test to identify compliance in studies lasting more than a week is compromised (Santo-Buelga et al. 2010). To illustrate, even if participants followed the protocol, abstinence from consuming the dose of chocolate for the last few days before the appointment might have not clearly identified their compliance. On the other hand, this test might provide positive results if participants mainly adhered to the protocol for the last few days before the appointment.

As a result, whether the non significant effect obtained was due to the masking of the changes in polyphenols in the urine or to the non compliance of participants remains unclear.

A point worth of note is that despite the significant difference in total polyphenols between PRDC and placebo DC within mixed model ANOVA ($p=0.01$), this did not affect the changes in these levels over time, and might be explained by the fact that baseline levels of urinary total polyphenols were initially different between the two groups.

*Cortisol, cortisone levels and the activity of 11 β HSD1*

Although BMI increased, it was not associated with a significant change in cortisol and cortisone levels or in the activity of 11-β HSD1. The results are consistent with the outcomes of Al Moosawi et al (2010; 2012) which showed no significant changes in glucocorticoid metabolism in the PRDC group. As mentioned in section 5.1.2, factors such as the implication of polyphenols other than flavanols in improving cortisol metabolism, and confounding factors such as fat and sodium intake, might have led to such outcomes.

Results do not correspond with the fact that an increase in food intake and body weight could lead to an increase in HPA activity and cortisol levels (Drake et al. 2005). However, the fact that the increase in energy intake and weight were not sufficient to induce an increase in HPA cannot be excluded. Lastly, the lack of compliance in providing urine samples might have also influenced the results.

#### 4.2.2.2 Section II: Discussion of feasibility outcomes for a larger study

The feasibility of a larger study was assessed by the evaluation of the integrity of Study II protocol, validity and clarity of questionnaires, acceptability of treatment, as well as compliance and drop out rates (Lancaster et al. 2002). Participants self-completed the forms and questionnaires. Information sheets were clearly stated and questions were comprehensible and did not necessitate further modifications.

For the questionnaires, important information regarding medical history and social habits were obtained, and data regarding physical activity were collected before, during and after the study. These data mainly aimed to assess eligibility criteria and to avoid potential confounders such as diet and exercise. Therefore, these questionnaires can be considered valid as they clearly measured what they were intended to measure. However, the increase in weight that did not correspond to an increase in energy intake in diet diaries, suggests that participants underreported their energy intake. Based on this, providing information on how to accurately report food intake in the diaries (e.g. mention all food ingredients, use measuring cups) might be helpful. In addition, a close monitoring to the diet of participants by a dietitian might need to be considered.

Furthermore, the food frequency questionnaire presented many potential limitations. First, as it was solely designed to identify differences in flavonoid intake throughout the study, only medium and high flavonoid content foods were considered (Appendix 9). Yet, some low flavonoid foods which were not included might have been consumed excessively, and have potentially affected the total flavonoid intake. In addition, some herbs, peppers and spices that are high in flavonoids (as mentioned in the USDA database (USDA 2011b)), were not mentioned due to their common low intake. Moreover, other foods also known to contain a high amount of flavonoids like lemons, elderberry, tangerines, raw spinach, sweet potatoes (USDA 2011b) were not included for different reasons such as their low probability of consumption while raw, and their intake in small quantities. Consequently, the validity of the current FFQ was compromised as it did not entirely assess flavonoid intake. In view of the strong correlation between FFQ and total flavonoids in the urine according to few studies (Rautiainen et al. 2008; Vian et al. 2013), a properly

designed FFQ could help in determining changes in polyphenol intake, can be used for a large study. A detailed FFQ might also be helpful in identifying seasonal changes that could affect flavonoid intake**,** and as a result, influence the study outcomes. For instance, Mink et al. (2007) developed a FFQ with 127 food items based on USDA data in which foods containing flavonoids were mentioned. Analysis of this FFQ was carried out by multiplying the amount of flavonoids (in mg) in one serving of food by the frequency of consumption per week. Results were then adjusted for energy intake. Designing a similar FFQ might be more precise in estimating the total amount of flavonoids in the diet, and could possibly provide information on the changes of flavonoid intake throughout the intervention in a larger trial.

The dose of dark chocolate (20g which provided 102 Kcal) and the amount of polyphenols (500mg of polyphenols) might possibly need to be tested on a large trial before considering adopting changes to the quantity of chocolate or to the dose of polyphenols. In fact, a lower amount of chocolate with a similar polyphenol dose might increase its bitterness and result in a decrease in compliance. Also, choosing a higher amount of polyphenols might increase complaints and side effects such as gastrointestinal discomfort (Mead 2007). Hence, a study well controlled for confounding factors (diet, exercise) might provide suggestions for any future changes in the dose of DC or in the amount of polyphenols based on the outcomes. Randomization procedure using sealed envelopes and the random allocation of chocolate was shown to be appropriate in randomizing participants.
As for the recruitment rate, one of the main limitations of this study was the difficulty in recruiting overweight participants with BMI>25 $Kg/m^2$. As stated in study I, it is crucial to identify barriers to the participation of the overweight population in research studies. In fact, this decrease in participation could lead to a reduction in the statistical power and efficiency of trials (IDRE 2014). The difficulty remains in the recruitment of participants who were not or had not been on a weight management program for two months prior to the study. This was mainly intended to identify the effect of DC on BMI in participants who follow their usual diet. Excluding participants on a diet program was mainly due to the different pace of

weight loss between individuals, which might make it difficult to identify the implication of PRDC in weight loss. Based on this, it was not possible to recruit volunteers from weight loss centres, as they do not meet the eligibility criteria. In addition, as some participants were reluctant to participate attributing the reason to geographical distance, offering incentives while involving a wider location should probably be considered.

The dropout rate observed in this study was 30 %. This rate matches with the results of a weight loss study by Drummond et al. (2004), and with the long term study conducted by Curtis et al. (2012) on dark chocolate; yet the latter study lasted for a year. This relatively high drop out rate should be taken into consideration in future recruitment strategies to avoid an underpowered study. In addition, only two participants reported complaints following PRDC consumption, suggesting few side effects of the treatment. Therefore, it would be possible to administer this product for a larger number of participants.

In this study, urine collection was intended to analyse compliance to intervention and antioxidant capacity resulting from daily PRDC consumption. To minimize burden, participants were not asked to provide a 24-hour urine sample before the follow-up appointment. However, this might have been important in identifying changes in flavonoid intake following the study, and would have provided a better overview of the compliance throughout the study (by comparing the levels of polyphenols/antioxidant capacity before and after the end of the study). Hence, including urine collection before the follow-up appointment should probably be included in a future protocol.

For the sample size analysis for a larger trial, the numbers presented should be adequate to conduct a future study with significance. The mid-pre intervention estimation was 260 participants, which is 419 % higher than the sample size estimated at post – pre point (62 participants), and 200 % higher than the post - mid point (130 participants). This difference could be explained by the non significant results obtained between the post – mid and mid – pre time points in the two groups,

which resulted in the need for a larger number of participants to detect an effect. Among the sample size options, it would probably be the most significant to go for the highest number of participants (338 participants while considering the drop out rate), yet the recruitment of such number might be impractical. Therefore, as suggested by Delucchi (2004), adopting a particular sample size in studies must be a compromise between scientifically suggested numbers and the available time and budget.

Lastly, designing a study with more frequent appointments (e.g every 2 weeks) might be more helpful in obtaining a better overview of the change in weight throughout the study.

In conclusion, results of this small scale study suggest that the daily consumption of a dark chocolate snack whether low or high in polyphenols led to an increase in weight. The potential underestimation of energy intake by this population rendered it difficult to identify changes in energy and macronutrient intakes after dark chocolate consumption. Results also suggest the feasibility of the current protocol for a large study with few modifications. As a result, snacking on a small amount of chocolate should remain in the context of a balanced diet in order to avoid its potential unfavourable effects on body weight.

# CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

The aim of this research was to assess the preventive effect of PRDC on risk factors for type 2 diabetes and CVD including IR, blood pressure, serum cholesterol levels and antioxidant and inflammatory markers. For this purpose, participants were recruited with no chronic diseases. For the two studies carried out, it was questioned whether participants should be identified with the term "healthy", due to the fact they have no history of CVD, type 2 diabetes and hypertension. However, because of the inclusion of normal weight and overweight participants, this identification might become inaccurate. In fact, as explained in section 1.1.2, overweight and obesity are correlated with more complications than normal weight, such as higher inflammatory state and oxidative stress (Silver et al. 2007); and obesity was even qualified as a disease (Mechanick et al. 2012). Hence, to avoid any underestimation of the overweight and obesity state, the term "healthy participants" was replaced by participants with no known hypertension, type 2 diabetes or CVD in both studies.

The difficulty in recruitment did not lead to the required number of participants in both studies. Reasons such as the need to perform venepuncture as a study requirement (study I), the long term commitment (Study II), the need to restrict flavonoid intake for the whole study period (Study I), the burden in reaching the research lab location and work commitment were noted by subjects. As an effort to improve recruiting, steps such as the increase in advertisement and the coverage of the transportation costs were considered.

The use of a relatively low calorie daily dose of chocolate in both trials (20g; 102 Kcal) resulted in presenting DC as an energy dense food. This has advantages over the studies of Di Renzo et al. (2013) and Grassi et al. (2005) which used a high dose of DC (100g; contained 561 Kcal and 515 Kcal, respectively); this amount of energy provides not less than 20 % and 30% of the daily usual energy intake of men and women, respectively (CDC 2011b), and might result in weight gain and other complications, particularly over the long term. Therefore, the amount of DC used in Studies I and II might have helped in minimizing the detrimental effects mainly

noted in the placebo group, and decreased the magnitude of the increase in weight, glucose, insulin and TG levels.

The favourable effects of PRDC on insulin sensitivity and serum oxidised LDL underline the importance of PRDC in preventive medicine due to the fact that it is cost effective. In fact, it was suggested that 50000$ could be saved per years of life when 42 $ per person per year is utilized to buy chocolate (Zomer et al. 2012). Given that the favourable effects of PRDC have been established, PRDC could be implemented in future recommendations to improve health, in a similar way as olive oil or nuts. On the other hand, findings of the placebo group (increase in BMI, insulin, IR, glucose and TG levels in study I, and the increase in BMI in study II) raise concerns over the different types of DC in the market, as the current methods of processing might cause a considerable loss of polyphenols (Cooper et al. 2008). This leads to the hypothesis that chocolate in the market might be detrimental to health. For marketing purposes, chocolate companies mainly aim to increase food palatability by masking the astringent flavour and increasing consumer acceptability. This results in the use of processing methods that might cause major polyphenol losses. It is then very likely that these companies are more eager to improve taste rather than preserving polyphenols. Qualitative data from studies I and II reinforce this idea that low polyphenol DC is more preferred than high polyphenol DC. Studies that analyse the content of polyphenols in DC in the market are crucial, and the need for a legislation regarding the polyphenol amount of chocolate should probably be considered, provided that the beneficial effects of PRDC on health have been established.

Furthermore, there are some doubts regarding the long term acceptability of the regular consumption of chocolate, particularly the one rich in polyphenols (due to its bitterness). The study of Ried et al. (2010) showed that 20% of participants found it hard to consume chocolate as a long term intervention, and suggested that chocolate might not be as practical as capsules. Moreover, with the adverse effects and complaints reported in studies I and II, data that support the long term intake of polyphenols are needed, and efforts towards improving the bitterness and the taste of PRDC should probably be considered.

A point worth mentioning is that in study I, the placebo DC resulted in an increase of TG (by 0.13 ± 0.23 mmol/l), BMI (by 0.17 ± 0.32 Kg/$m^2$), fasting insulin levels (by 0.77 ± 1.56 µIU/ml), HOMA-IR (by 0.27 ± 0.44) and glucose levels (by 0.44 ± 1.08 mmol/l). Although results are statistically significant, there is a question about the clinical relevance of these outcomes. Levels of TG levels, albeit increased (by 15%) remained within normal levels. For glucose, levels slightly increased, and the possibility of day-to-day fluctuations in glucose levels cannot be excluded (Sacks 2011). In addition, after adjusting for baseline imbalances (via ANCOVA), the effect of placebo DC on glucose levels was no longer significant (p=0.29). With regards to weight, the increase in 1 Kg was reported to be clinically significant (Jousilahti et al. 1996), thus the increase in body weight might not be considered clinically relevant in this study. In view of the small changes from baseline in glucose, triglyceride and BMI levels, it cannot be ruled out that few participants in the placebo group did not abide by the recommendations to attend the appointments while fasting. As for the PRDC group, the reductions in HOMA-IR were small (by 0.2 ± 0.34), compared to the studies of Grassi et al. (2005) (HOMA-IR decreased by 0.94 ± 0.42) and Al Moosawi et al. (2012) (HOMA-IR decreased by 1.03 ± 0.76). Therefore, results obtained constitute a basis for carrying out more studies investigating the long term effects of PRDC and low polyphenol DC on anthropometric, biochemical and physiological markers.

Although the aim of both studies was to recruit participants from the whole population, there should be a consideration towards including post menopausal women, particularly in Study II. As previously discussed, post menopausal women undergo losses in lean body mass and have differences in body composition and fat distribution, compared to pre-menopausal women. This leads to an increase in the ratio of fat mass/ lean mass particularly in the lower part of the body, which affects BIA measurement (since BIA electrical current passes through the legs) (Dehghan and Merchant 2008). Studies that include exclusively postmenopausal women might be helpful in assessing the effect of PRDC consumption in this category.

The reported diet diaries in both studies showed a potential underreporting. This was mainly explained by the increase in weight that was not supported by an increase in

food intake in the diaries. Thus, it would be important to improve the way of estimating the actual daily intake, while considering the practicality of the method, and the fact that it does not impose a big effort from participants. In the last few years, methods using smart phone devices have been shown to be useful. For instance, researchers developed a smart phone application called “Recaller” as a nutritional assessment tool (Suzuki et al. 2012). This application constitutes an easy method to keep photo records of all consumed foods. Although this method does not solve the problem of limiting food intake during an intervention (the fact of recording may alter diet (Lee and Nieman 2007)), it reduces the error of assessing food portions by individuals and decreasing intake to avoid the burden of reporting. This should be helpful in obtaining a better estimate of the actual food and energy intake in future studies.

It is important to mention that in view of the increase in TG, glucose, insulin and BMI levels in the placebo group after 4 weeks (study I), and the increase in BMI in the second study, there is a question about the ethical implication of daily administering dark chocolate in long term studies (such as 12 weeks), particularly the one deficient in polyphenols. Therefore, an information sheet that mentions the potential side effects of DC, particularly for the control group should be considered in future trials. Furthermore, the restriction of polyphenol/flavonoid intake as a study requirement might need to be considered. This restriction could be problematic particularly in studies lasting for a long period (12 weeks), due to the favourable effects of polyphenols/flavonoids on health (Stevenson and Hurst 2007).

### 5.1.1 Limitations

Given that the aim of the studies was to determine the effect of PRDC consumption in the general population, while recruiting normal weight and overweight participants, it was not possible to reach the desired number of overweight participants in both studies, due to the fact that they were reluctant to participate. As this study did not offer incentives apart from the free chocolate, there was no great interest for this weight category to take part. A study that looks at the reasons of

reluctance of the overweight category to participate in research studies might be helpful in increasing compliance among this population. As far as we know, this unwillingness was not previously described in the literature.

In addition, women represented the largest part of both studies, which might have limited generalization of the results. Other factors such as the considerably young population and the predominance of students, were mainly due to the location of the studies, and might have also affected study generalization. Furthermore, there was no randomization according to age or gender. In study I, there was a significant age difference between normal weight and overweight participants in the placebo group ($p= 0.034$). A meta-analysis previously reported a greater effect of cocoa/DC on blood pressure when participants were younger (Ried et al. 2012). Hence, randomization would have been more reliable in eliminating any possible confounding factors. The difficulty arises from the small number of participants, which made it impractical to randomize participants according to the above mentioned criteria.

Moreover, since there were no restrictions made on the time and frequency of eating the daily DC dose in the two studies, inter-individual and intra-individual variations in response to intervention arising from consuming DC at fasting or with meals cannot be excluded. This is particularly important if the main mechanism by which PRDC improves IR and reduces weight, involves its effect on inhibiting digestive enzymes (Gu et al. 2011) and increasing GLP-1 in the gut (Dorenkott et al. 2014). These differences might have caused a considerable impact on the outcomes.

Similarly to all dietary intervention studies, an inability to control the diet of participants resulted from the inclusion of free-living individuals who exhibit a large variability in their dietary habits (Monsen and Van Horn 2008). Therefore, it was not possible to verify whether participants consumed the dose of chocolate daily as required. Also, the day-to day fluctuations in the diet made it difficult to determine the true effect of cocoa polyphenols on anthropometric and physiological markers. Furthermore, the possibility that participants did not adhere to the dietary protocol,

and did not maintain their regular diet throughout the intervention cannot be excluded. Therefore, unless studies are controlled for food intake and physical activity, many changes in the diet could mask the effects of DC intervention.

Another limitation is the large inter-individual variability in the metabolism of polyphenols (Santos-Buelga et al. 2010), which is due to the difference in the colonic microflora between individuals (D'arhivio et al. 2007). As it is an oral dose, it is hard to determine the absolute availability of polyphenols as it is the case of injected doses. The difference in polyphenol metabolism was estimated to be around 15.8 % between individuals (Rodriguez-Mateos 2014).

Moreover, both studies consisted of taking baseline and post-intervention data (baseline, mid- and post-intervention data in study II), each on one single occasion. Therefore, routine measurements were not considered. Although this protocol is common in most of the studies (examples: Engler et al 2004; Baba et al 2007a; Al Moosawi et al 2012), it is affected by the daily fluctuations in many parameters, and particularly body weight and blood glucose levels. Despite the fact that participants were asked to maintain their usual diet, it was not possible to control the changes in weight resulting from energy fluctuations in the past few days preceding the appointments. Measurement of these parameters daily would have been more reliable in detecting changes caused by DC supplementation. For instance, Grassi et al. (2005) monitored blood pressure daily for the whole study period (15 days). However, due to the longer term of the conducted studies (4 weeks and 16 weeks) and the burden on participants, it was not practical to assess these parameters routinely.

Lastly, the adoption of a parallel study design, despite it advantages, presents few limitations in terms of inter participant variation, since the participant does not serve as his own control. This might lead to a decrease in the power of studies (Monsen and Van Horn 2007) (Section 3.3.4)

#### 5.1.1.1 Study I

Some limitations specific to this study were noted. The compliance to intervention was not assessed by analysing total polyphenols in a 24-hour urine sample as a biochemical marker for compliance. Despite the limitations related to this test (section 3.4.6.2), it might have provided a better idea of adherence. Asking participants whether they consumed the chocolate, and counting the empty containers were the only means to assess adherence to daily DC consumption. Nevertheless, previous studies have also assessed compliance similarly to this study (Grassi et al. 2005; Davison et al 2008; Muniyappa et al. 2008). The collection of urines samples would have also been useful for the analysis of the urinary $F_2$ isoprostanes concentration, since the latter parameter represents a direct measure of oxidised LDL in the urine (Jialal 1998).

Another limitation is the duration of the study (4 weeks), which might have been insufficient to identify the effects of PRDC on some parameters such as blood pressure levels. Furthermore, the fact that parameters were assessed at 2 appointments (baseline and week 4) might have been less reliable than measuring these parameters at more frequent appointments (e.g. week 2, week 4 and week 6).

Moreover, the sample size did not provide enough power to explore the effect of other factors such as age and gender on the results, as some studies showed their impact on the outcomes. While a meta-analysis reported a possible influence of age on the effects of cocoa/DC on blood pressure (Ried et al. 2013), a new research suggested a minimal implication of age on the metabolism of flavanols (Rodriguez-Mateos 2014). Furthermore, a study suggested than males better responded to the LDL oxidation lowering properties of cocoa polyphenols than females (Ibero-Baraibar et al. 2014).

This study did not also help to make strong conclusions regarding the impact of BMI on the outcomes. In addition, the findings are limited by the lack of knowledge of the physiological mechanisms underlying the improvement of insulin resistance and

oxidised LDL, and which polyphenols or polyphenol metabolites were responsible for these effects.

#### 5.1.1.2 Study II

The main drawback of this study is the small sample size, hence results are mainly preliminary. Although expected, the high dropout rate (30 %) probably affected the power of the study. In addition, the food frequency questionnaire used was not entirely reliable in assessing flavonoid intake of participants during the intervention, as it lacks foods with low content of flavonoids. Furthermore, this FFQ did no take into consideration the combination foods (such as mixed vegetables and pizza). Also, as it was assumed that foods that are not in the USDA table (for foods rich in flavonoids) do not contain flavonoids, some other databases need to be checked while developing a more accurate FFQ. Moreover, the fact that the intake of flavonoids was not restricted during the study might have masked the changes in total phenols and antioxidant capacity in the urine resulting from PRDC consumption.

Another limitation is the decrease in compliance because of the long term nature of the study (12 weeks) which might have led to a decrease in compliance. Low compliance was described as the major threat to the validity in trials (Swinscow and Campbell 2002). Moreover, there was no blood sample test taken to evaluate the long term effect of DC on several biochemical and physiological markers (such as insulin, oxidised LDL and lipid levels). This would have been helpful to determine any beneficial/detrimental effects on these markers resulting from PRDC/ placebo DC consumption, and their correlation with body weight.

Also, the estimation of energy expenditure via BIA machine did not provide an accurate measurement of this parameter, and did not help to assess the actual change in BMR resulting from a change in oxygen consumption and carbon dioxide production throughout the intervention.

Furthermore, there was no measurement of polyphenol and antioxidant capacity in the urine before the follow-up appointment. This could have been useful in assessing

any differences in polyphenol intake after the study, and in providing additional data about compliance. The evaluation of F2 isoprostanes in the urine as a direct measurement of LDL oxidation (Jialal 1998) might have also been helpful in assessing the antioxidant effects of PRDC.

Moreover, measurement of hip circumference as a part of anthropometric measures would have been helpful in assessing the body adiposity index, which can be obtained from height and hip circumference, and is highly correlated to fat mass (Bergman et al. 2011). This would have provided another measure of adiposity.

Finally, even though efforts towards the estimation of underreporting energy intake were considered in this study (by comparing energy intake to energy expenditure), underreporting was evaluated through non validated methods such as physical activity levels, which were described by participants. In addition, BMR was estimated via BIA machine, which presents important drawbacks cannot also be excluded (Dehghan and Merchant 2008). This might have affected the estimation of the total energy expenditure of participants.

### 5.1.2 Directions for future research

The beneficial effects of PRDC on insulin sensitivity and LDL oxidation suggest conducting more trials to elucidate these effects, while targeting the general population. Determining the optimal dose and duration responsible for decreasing risk factors for type 2 diabetes and cardiovascular risk factors, along with identifying the types of flavonoids responsible for causing a favourable effect are needed.

It would also be crucial to investigate the mechanisms involved in the effect of cocoa polyphenols on insulin responses and oxidative stress. Therefore, studies that assess NO levels/NOS enzymatic activity might be helpful. In addition, as AMPK pathway was regarded as a principal regulator of lipid and glucose metabolism (Ali et al. 2014), testing its effects in addition to PI3K/AKT pathway, will help to understand the molecular mechanisms underlying the effect of PRDC on obesity and its related diseases.

In addition, it would be important to identify the receptors to which flavonoids/flavanols bind to induce a beneficial effect. In fact, the enhanced oxidative capacity in muscles of mice due to epicatechin (Nogueira et al. 2011) might suggest the presence of receptors for this monomer in muscles. Another study stated that epicatechin works via binding to opioid receptors resulting in cardiac protection in mice (Panneerselvam et al. 2010). Knowledge of these receptors will constitute an important step towards the identification of the mechanisms of action of these flavanols.

Because of the limited number of participants in Study II, larger trials examining the link between PRDC and body weight are needed. Research that considers many aspects of weight control such as satiety, fatty acid synthesis macronutrient absorption and thermogenesis are needed. This includes looking at satiety hormones, evaluating visual analogue scales, assessing mitochondrial biogenesis, LBM and body fat percentage as well as measuring energy expenditure via indirect calorimetry. Moreover, testing the long term effect of PRDC (12 weeks) on glucose and insulin levels is crucial, due to the possible link between improving insulin resistance and decreasing BMI through improvement of insulin signaling pathways (such as PI3K/Akt).

It is also crucial to ensure that the placebo DC and the intervention DC fairly match for all components (methylxanthine, magnesium, cocoa butter), in order to eliminate possible confounding factors. Also, human studies should be well controlled for diet and physical activity. This will reduce confounding effects resulting from changes in energy intake or physical activity throughout the intervention

Given the potential beneficial effects of PRDC and the detrimental effects of placebo DC, a crucial future step would be the analysis of the total polyphenol of DC in the UK market. This should constitute a direction towards stating the amount of polyphenols as a part of a labelling requirement. In addition, efforts towards uniting the processing methods of cocoa might need to be considered. Also, because of the lower acceptability of DC rich in polyphenols compared to DC low in polyphenols,

and the possible adverse effects of fat and sugar in chocolate, efforts should be made to deliver a high-flavonoid chocolate in a low sugar and low fat content, while increasing its acceptability and decreasing its bitterness.
In view of the potential side effects noted, there is a need for data regarding the long-term safety of chocolate components (flavanols, theobromine etc...) before recommending the intake of this snack regularly. It would be also worth taking into account the control of the polyphenol content of milk chocolate in view of its wide consumption, as the study of Fraga et al. (2005) showed a significant effect of flavanol-rich milk chocolate on serum lipids and blood pressure.

Studies should also specify the way polyphenols have been assessed in research, because of the potential differences in the estimation based on different methods. This will allow a more accurate comparison between studies.
Moreover, in order to improve the practicality of chocolate as a treatment option, testing the frequent but not daily intake of dark chocolate (e.g. 3-4 times a week) might be a possible option to monitor the long term intake of DC. In addition, in view of the difficulty in recruitment, efforts towards improving recruitment strategies should probably be considered.

Lastly, the drawbacks of BMI in identifying some normal weight individuals at high risk of CVD (Di Renzo et al. 2013), and overweight individuals with high lean body mass (Snijder et al. 2006) underline the need for other anthropometric measures to categorize the population at risk. This will be helpful in order to implement appropriate prevention strategies.

### 5.1.3 General Conclusion

This research aimed to determine the effect of PRDC on anthropometric, biochemical, dietary and physiological markers in normal weight and overweight adults. Study I is, as far as we know, the fifth human study that analysed the effect of PRDC on IR/insulin sensitivity in individuals with no diabetes type 2, hypertension

or CVD. Study II provided preliminary human data on the long term effect of PRDC on body weight and body composition.

The results showed that consuming 20g of DC rich in polyphenols (500 mg) for a period of four weeks exerted lowering properties on insulin resistance and oxidised LDL levels, both considered potential risk factors for type 2 diabetes and CVD. Yet, because of the complexity of polyphenols, there is a lack of knowledge of the mechanisms of action involved in improving IR and oxidative stress. Results underline the possible implication of PRDC in preventive medicine due to the fact that it is cost effective. Findings suggest that polyphenols, and particularly flavanols, might be promising dietary components for the prevention of type 2 diabetes and CVD risk factors, and PRDC might be subsequently considered a nutraceutical for helping in the prevention or delay of the onset of type 2 diabetes, without causing the side effects of medications. This raises the need for further investigations in order to make future recommendations.

The non-significant effects of PRDC on blood pressure, serum lipid levels and hs-CRP noted in study I, and on glucocorticoid metabolism in studies I and II suggest that 1) the marketing about the preventive effects on PRDC on blood pressure might need to be reconsidered 2) PRDC might not constitute a source of prevention against dyslipidemia 3) PRDC might not exert a favourable effect on glucocorticoid metabolism 4) PRDC might not decrease inflammation. Results could be attributed to the fact that participants did not have diabetes, CVD or hypertension. This made them less likely to be affected by the intervention. Further long term studies controlling for potential confounding factors are needed to refute the preventive effect of PRDC on the above mentioned parameters.

Daily supplementation of DC (20g) for 12 weeks increased BMI in both placebo DC and PRDC groups. This increase in weight was not accompanied with significant changes in waist circumference, body composition, urinary glucocorticoid hormones and antioxidant capacity. Findings are limited by the small sample size of the study. Results suggest the importance of considering the energy and fat content of DC, and the introduction of this snack in a controlled calorie diet to avoid its adverse effects.

Further studies aiming to determine the effect of PRDC on BMI in humans are needed.
The preliminary results provided by study II suggest the feasibility of carrying out a large scale study with a similar protocol that investigates the effect of PRDC on body weight. Few modifications such as improving the reliability of FFQ and closely monitoring the participant's diet were proposed.

The increase in BMI, TG, insulin resistance and glucose in the placebo DC group in study I but not in the PRDC group, suggest that polyphenols may counteract the negative effects of the high fat and energy in chocolate. However, the increase in BMI occurred in the placebo DC and PRDC groups following 12 weeks of intervention in Study II. The latter outcome might be attributed to the adaptation to the quantity of polyphenols, which could limit the ability of these components 3to counteract the adverse effects of fat in the diet over the long term. Further studies including a large population might elucidate this effect.

The placebo DC adverse effects on BMI, TG, insulin resistance and glucose levels suggest that the analysis of the polyphenol content in the market is necessary. Controlling polyphenol intake might start by unifying cocoa processing as a first step to include this product in public health recommendations, and ending towards regulating and labelling the chocolate with polyphenol or flavonoid content.

The potential underreporting of food intake in the studies did not allow an accurate identification of fluctuations in energy intake throughout the study. This suggests that reporting of diet diaries should be improved via the instruction of participants on how to properly document food intake, and the introduction of photo records as a way to improve the estimation of food portions. In addition, the difficulty in recruiting overweight participants suggests the need to improve recruitment strategies in order to increase the power of future studies.

Lastly, because of the lower acceptability of DC rich in polyphenols compared to DC low in polyphenols, efforts should be made to increase the acceptability of PRDC.

Testing the frequent but not daily intake of dark chocolate (such as 3-4 times a week) might be a better option for the long term intake of DC.

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## CHAPTER 7: APPENDICES

### Appendix 1: Study I Information sheet and consent form for potential participants

Queen Margaret University
EDINBURGH

Information Sheet for Potential Participants

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON INSULIN SENSITIVITY IN NORMAL WEIGHT AND OVERWEIGHT ADULTS

You are invited to take part in this research study. Please read carefully all the information before you decide to participate.

My name is Grace Farhat and I am a PhD student in public health nutrition from the school of health sciences at Queen Margaret University in Edinburgh. I am currently investigating the effects of dark chocolate rich in polyphenols on different markers responsible for the occurrence of several diseases.

Background

Polyphenols are antioxidants widely available in foods. Polyphenol-rich foods -and dark chocolate in particular- have been the subject of research of many studies in the past few years, focusing on their role in reducing risk factors for type 2 diabetes and cardiovascular diseases. Thus, determining the beneficial effect of polyphenols in dark chocolate on insulin sensitivity and other health markers might help in decreasing the occurrence of chronic diseases.

Procedure

If you agree to participate in the study, you will be advised to follow your usual diet, and to substitute dark chocolate for another food usually consumed. The researcher may help you with properly replacing dark chocolate instead of other foods. You will be also asked to avoid eating high quantities of foods rich in flavonoids like red wine, blackberries, blueberries and tea (a list of flavonoid-rich foods will be provided to you), one week before the start, and subsequently for the duration of the study. You will also be requested to avoid drinking alcohol 24 hours before the visit, to avoid heavy physical activity 12 hours before the visit, and to consume the last dose of dark chocolate 12 hours prior to the second visit. You will also be requested to be on a fasting state when attending the 2 appointments. To evaluate your diet, you will be asked to complete a three-day diet diary twice during the intervention.

You will randomly receive 20g of one of the 2 types of dark chocolate (placebo low in polyphenols, or dark chocolate rich in polyphenols), daily for a period of 4 weeks. Before each appointment, you'll be asked to collect 2 saliva samples for the measurement of cortisol and cortisone levels. A blood test will be performed during each of the two visits for the measurement of total cholesterol, HDL, LDL, Triglycerides, insulin, glucose, C-reactive protein (a marker of inflammation), and oxidised LDL levels.
Your blood pressure will be measured using a digital sphygmomanometer, and you will have your height, weight, waist circumference and BMI measured as well.

Duration of the study
This study is expected to last for 4 weeks. You will be required to attend 2 appointments during this period, each appointment will require 25-30 minutes of your time.

Risks
There are no risks related to this research.

Benefits

The dark chocolate consumed may improve your health and biological markers. You will receive the dark chocolate free of charge. In addition, you will receive a copy of the results of all the tests performed if you wish.

Confidentiality

Your name will be replaced with a participant identification number, and it will not be possible for you to be identified in any reporting of the data gathered. All data collected will be anonymous. The results may be published in a journal or presented at a conference.

You'll be free to withdraw from the study at any time without giving any reason.

If you would like to contact an independent person, who knows about this project but is not involved in it, you are welcome to contact Dr Iain Gow. His contact details are given below.

If you have read and understood this information sheet, and all your questions have been answered, and you would like to be a participant in the study, please sign the consent form.

Contact details of the researcher


Name of researcher: Grace Farhat

Address: PhD student
Public Health Nutrition
Department of Dietetics, Nutrition & Biological Sciences
Queen Margaret University, Edinburgh
Musselburgh, East Lothian
EH21 6UU

Email/ Telephone: gfarhat@qmu.ac.uk / 0131 474 0000

Contacts details of the supervisor


| | |
|---|---|
| Name of supervisor: | Dr Emad Al-Dujaili |
| Address: | Senior Lecturer in Biochemistry |
| | Department of Dietetics, Nutrition & Biological Sciences |
| | Queen Margaret University, Edinburgh |
| | Musselburgh, East Lothian |
| | EH21 6UU |
| Email/ Telephone: | ealdujaili@qmu.ac.uk / 0131 474 0000 |


Contact details of the independent adviser


| | |
|---|---|
| Name of adviser: | Dr Iain Gow |
| Address: | Lecturer in microbiology |
| | Department of Dietetics, Nutrition & Biological Sciences |
| | Queen Margaret University, Edinburgh |
| | Musselburgh, East Lothian |
| | EH21 6UU |
| Email/ Telephone: | igow@qmu.ac.uk / 0131 474 0000 |

Queen Margaret University
EDINBURGH

PARTICIPANT CONSENT FORM

Participant Identification number for this study:

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON INSULIN SENSITIVITY IN NORMAL WEIGHT AND OVERWEIGHT ADULTS

I confirm that I have read and understood the information sheet and this consent form.

I have had the opportunity to ask questions, and have had all my questions answered satisfactorily.

I understand that I am free to withdraw at any time without giving any reason.

I hereby voluntarily agree to take part in the above study

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher:


| | |
|---|---|
| Name of researcher | Grace Farhat |
| Address: | PhD student<br>Public Health Nutrition<br>Department of Dietetics, Nutrition & Biological Sciences<br>Queen Margaret University<br>Musselburgh<br>EH21 6UU |
| Email/Telephone: | gfarhat@qmu.ac.uk / 0131 474 0000 |

**Appendix 2: General questionnaire for Study I**

Queen Margaret University
EDINBURGH

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON INSULIN SENSITIVITY IN NORMAL WEIGHT AND OVERWEIGHT ADULTS

QUESTIONNAIRE

Participant Identification Number: Date:

Date of Birth: / /19 Age:

Sex: Male ☐ Female ☐

Occupation:

Ethnicity: ☐ European ☐ South Asian
☐ Chinese ☐ Ethnic South /Central American
☐ Japanese ☐ Sub-Saharan Africans
☐ Eastern Mediterranean /Middle East Arab
☐ Other (please specify)…….

A. Medical and Family History

| 1. Do you have any of the following diseases: | Yes | No |
|---|---|---|
| High Blood Pressure | ☐ | ☐ |
| Cardiovascular diseases | ☐ | ☐ |
| Diabetes | ☐ | ☐ |
| Stroke | ☐ | ☐ |

2. Do you take any medications for: Yes ☐ No ☐

High Cholesterol level

If yes, please specify the type and brand name

……

……

……

High Triglycerides level ☐ ☐

If yes, please specify the type and brand name

……

……

……

Hypertension ☐ ☐

If yes, please specify the type and brand name

……

……

……

Diabetes ☐ ☐

If yes, please specify the type and brand name

……

……

……

Any other conditions ☐ ☐

If yes, please specify the condition and brand name of the medication

……

……

……

3. Do you take any dietary supplements? Yes ☐ No ☐

If yes, please specify the type, brand name and frequency of intake

……

……

……

……

4. For women only

Are you on a Hormonal Replacement Therapy? Yes ☐ No ☐

Do you take contraceptives? Yes ☐ No ☐

If yes, Please specify the type

……

……

5. Are there any chronic diseases in your family such as diabetes, hypertension and/or heart diseases? ☐ Yes ☐ No

If yes, please specify:

……

……

……

B. Personal habits:

6. Do you currently smoke cigarettes, pipe or cigar?

Yes, daily ☐

No ☐

Occasionally ☐

(Less than one cigarette, pipe or cigar per day)

7. Do you consume alcohol?

Yes, daily ☐

Please specify what kind(s) of alcoholic beverage(s) you drink and the amount daily consumed

……

……

No ☐

Occasionally ☐

8. Do you exercise? Yes ☐ No ☐

Types of exercise:

……

……

……

-------hour(s) -------day

------week

------month

9. Do you usually consume dark chocolate: ------pieces -----day

-----week

-----month

Do you usually drink cocoa -------cups -----day

-----week

-----month

10. Have you been recently (in the past 2 months) or are you currently on a weight management program?

Yes ☐ No ☐

**Appendix 3: Physical activity questionnaire for Study I**

Physical Activity questionnaire (to be filled during the second appointment)

Did you exercise during this month? Yes ☐ No ☐

Types of exercise:

……

……

-------hour(s) ----------day

--------week

-------month

**Appendix 4: Acceptability of treatment questionnaire for study I**

Acceptability of treatment

1. How did you find consuming dark chocolate for 4 weeks:
   a. Very acceptable
   b. Somewhat acceptable
   c. Unacceptable

Reasons for unacceptability:

……

……

……

Are you willing to continue having dark chocolate frequently?

Yes ☐ No ☐

Comments:

……

……

……

**Appendix 5: Diet diaries form**

*Participant Identification number:*

Please use the forms below to write down all the foods and beverages consumed during 3 typical days (preferably 2 weekdays and one weekend)

| | Time | Food/Drink | Brand name (if applicable) | Amount consumed |
|---|---|---|---|---|
| Day 1 | | | | |

| Day 2 | Time | Food/Drink | Brand name (if applicable) | Amount consumed |
|---|---|---|---|---|
| | | | | |

| | Time | Food/Drink | Brand name (if applicable) | Amount consumed |
|---|---|---|---|---|
| Day 3 | | | | |

**Appendix 6: List of foods rich in flavonoids**

| |
|---|
| **Fruits** : berries (blueberries, blackberries, strawberries, raspberries, cranberries), oranges, apricots, bananas, cherries, plums, blackcurrants, black & red grapes, apples, figs, grapefruit, peaches and pears.<br>**Fruit juices**: Pure apple, pear, grape, grapefruit, pomegranate, lemon, orange, raspberry, strawberry, blackberry and cranberry juices |
| **Vegetables:** aubergine, red cabbage, rhubarb, artichoke, asparagus, brocolli, brussel sprouts, celery hearts, onions, sweet potatoes, Soja and soja products |
| Coffee |
| Tea (green tea, black tea) |
| Red wine |
| Dark chocolate, cocoa powder |
| Broadbeans |
| Hazelnut, chestnut |
| Apple sauce |

*List adapted from USDA database of the flavonoid content of selected foods (USDA 2011a). The list was provided to participants during the run-in phase period*

**Appendix 7: Study II Information sheet and consent form for potential participants**

Queen Margaret University
EDINBURGH

Information Sheet for Potential Participants

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON BODY WEIGHT IN OVERWEIGHT AND OBESE ADULTS

You are invited to take part in this research study. Please read carefully all the information before you decide to participate.
My name is Grace Farhat and I am a PhD student in public health nutrition from the School of health sciences at Queen Margaret University in Edinburgh. I am currently investigating the effects of polyphenols (in dark chocolate) on different markers responsible for the occurrence of several diseases.

**Background**

Polyphenols are antioxidants widely available in foods. Polyphenol-rich foods -and dark chocolate in particular- have been the subject of research of hundreds of studies in the past few years, most of these focused on the role of polyphenols in reducing risk factors for type 2 diabetes and cardiovascular diseases. Recent studies have discussed the possible implication of polyphenol-rich dark chocolate in weight loss through different mechanisms, thus determining its effect on body weight might be helpful in possibly including dark chocolate in weight loss diets.

**Procedure:**

If you agree to participate in the study, you will be advised to follow your usual diet, while substituting dark chocolate for another food usually consumed. The researcher may help you with properly replacing dark chocolate instead of other foods.

You will also be asked to avoid consuming cocoa-rich products or any other type of dark chocolate during the whole study period. Additionally, you will be requested to: 1) Be on a fasting state when attending the four appointments, 2) Avoid heavy physical activity 12 hours before each appointemtn, 3) Refrain from consuming caffeine-rich beverages (like tea and coffee) 12 hours prior to each appointment, and 4) Avoid alcohol consumption 24 hours before each visit. To evaluate your diet, you will be asked to complete a three-day diet diary and a food frequency questionnaire four times throughout the intervention.

You will randomly receive 20g of one of the 2 types of dark chocolate (placebo, or dark chocolate rich in polyphenols), daily for a period of 12 weeks.
Before each appointment, you will be asked to collect a 24-hour urine sample (to analyse cortisol, cortisone, total polyphenols and total antioxidant capacity in the urine) and you will have your height, weight, waist circumference and body composition (body fat percentage and lean body mass) measured during each visit.

**Duration of the study**

This study is expected to last for 12 weeks. You will be asked to attend 3 appointments during this period, and one follow-up appointment on week 16. Each appointment will require no more than 15-20 minutes of your time.

**Risks**

There are no risks related to this research.

**Benefits**:

The dark chocolate consumed may improve your health and biological markers. You will receive the dark chocolate free of charge. In addition, you will receive a copy of the results of all the tests performed if you wish.

**Confidentiality:**

Your name will be replaced with a participant identification number, and it will not be possible for you to be identified in any reporting of the data gathered. All data collected will be anonymous. The results may be published in a journal or presented at a conference.

You'll be free to withdraw from the study at any time without giving any reason.

If you would like to contact an independent person, who knows about this project but is not involved in it, you are welcome to contact Dr Iain Gow. His contact details are given below.

If you have read and understood this information sheet, and any questions you had have been answered, and you would like to be a participant in the study, please sign the consent form.

Contact details of the researcher:

Name of researcher: Grace Farhat

Address: PhD student
Public Health Nutrition
Department of Dietetics, Nutrition & Biological Sciences
Queen Margaret University, Edinburgh
Musselburgh, East Lothian
EH21 6UU

Email/ Telephone: gfarhat@qmu.ac.uk / 0131 474 0000

Contacts details of the supervisor:

Name of supervisor: Dr Emad Al-Dujaili
Address: Senior Lecturer in Biochemistry
Department of Dietetics, Nutrition & Biological Sciences
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Contact details of the independent adviser

Name of adviser: Dr Iain Gow
Address: Lecturer in microbiology
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Queen Margaret University

EDINBURGH

# PARTICIPANT CONSENT FORM

Participant Identification number for this study:

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON BODY WEIGHT IN OVERWEIGHT AND OBESE ADULTS

I confirm that I have read and understood the information sheet and this consent form.

I have had the opportunity to ask questions, and have had all my questions answered satisfactorily

I understand that I am free to withdraw at any time without giving any reason

I hereby voluntarily agree to take part in the above study

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher:

Name of researcher: Grace Farhat
Address: PhD student
Public Health Nutrition
Department of Dietetics, Nutrition & Biological Sciences
Queen Margaret University
Musselburgh
EH21 6UU
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**Appendix 8: General questionnaire for Study II**

Queen Margaret University
EDINBURGH

STUDY II: EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON BODY WEIGHT IN OVERWEIGHT AND OBESE ADULTS

QUESTIONNAIRE

---

Participant Identification Number: Date:

Date of Birth: / /19 Age:

Sex: Male ☐ Female ☐

Occupation:

Ethnicity: ☐ European ☐ South Asian

☐ Chinese ☐ Ethnic South /Central American

☐ Japanese ☐ Sub-Saharan Africans

☐ Eastern Mediterranean /Middle East Arab

☐ Other (please specify)…….

C. Medical and Family History

1. Do you have any of the following diseases:

| | Yes | No |
|---|---|---|
| High Blood Pressure | ☐ | ☐ |
| Cardiovascular diseases | ☐ | ☐ |
| Diabetes | ☐ | ☐ |
| Stroke | ☐ | ☐ |

2. Do you take any medications for:

| | Yes | No |
|---|---|---|
| High Cholesterol level | ☐ | ☐ |

If yes, please specify the type and brand name

……

……

……

| | | |
|---|---|---|
| High Triglycerides level | ☐ | ☐ |

If yes, please specify the type and brand name

……

……

……

| | | |
|---|---|---|
| Hypertension | ☐ | ☐ |

If yes, please specify the type and brand name

……

……

……

| | | |
|---|---|---|
| Diabetes | ☐ | ☐ |

If yes, please specify the type and brand name

……

……

……

| | | |
|---|---|---|
| Any other conditions | ☐ | ☐ |

If yes, please specify the condition and brand name of the medication

……

……

……

3. Do you take any dietary supplements? Yes ☐ No ☐

If yes, please specify the type, brand name and frequency of intake

……

……

……

……

……

4. For women only

Are you on a Hormonal Replacement Therapy? Yes ☐ No ☐

Do you take contraceptives? Yes ☐ No ☐

If yes, Please specify the type

……

……

……

5. Are there any chronic diseases in your family such as diabetes, hypertension and/or heart diseases? Yes ☐ No ☐

If yes, please specify:

……

……

……

……

……

6. Personal habits:

Do you currently smoke cigarettes, pipe or cigar?

Yes, daily ☐

No ☐

Occasionally ☐

(Less than one cigarette, pipe or cigar per day)

7. Do you consume alcohol?

Yes, daily ☐

Please specify what kind(s) of alcoholic beverage(s) you drink and the amount daily consumed

……

……

……

No ☐

Occasionally ☐

8. Do you exercise? Yes ☐ No ☐

Types of exercise:

……

……

-------hour(s) ----------day

-------week

------month

9. Do you usually consume dark chocolate: ……..pieces ---------day

---------week

--------month

Do you usually drink cocoa …….cups ----------day

-------week

----- month

10. Have you been recently (in the past 2 months) or are you currently on a weight management program?

☐ Yes ☐ No

**Appendix 9: Food Frequency questionnaire for Study II**

EFFECTS OF POLYPHENOL-RICH DARK CHOCOLATE ON BODY WEIGHT IN OVERWEIGHT AND OBESE ADULTS

FOOD FREQUENCY QUESTIONNAIRE

This questionnaire asks you some information about what you eat and is mainly designed to assess your polyphenol intake throughout the study.
Please answer all the questions and try to be the most precise possible.
If you need any clarification, please ask the researcher

YOUR DIET:

Please tick in the appropriate box (One answer per line)

| FOODS AND AMOUNTS | AVERAGE INTAKE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fruits | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
| Berries (Blueberries, blackberries, raspberries, cranberries, strawberries) ½ cup | | | | | | | | | |
| Orange (1 medium) | | | | | | | | | |
| Apple (1 medium) | | | | | | | | | |
| Apricots (3 pieces) | | | | | | | | | |
| Cherries (10 pieces) | | | | | | | | | |
| Plums (2 pieces) | | | | | | | | | |
| Black & Red grapes (10 pieces) | | | | | | | | | |
| Banana (1 medium) | | | | | | | | | |
| Figs (2 pieces) | | | | | | | | | |
| Grapefruit (half) | | | | | | | | | |
| Peach (1 medium) | | | | | | | | | |
| Pear (1 medium) | | | | | | | | | |
| Blackcurrants (1/4 cup) | | | | | | | | | |

| Foods AND AMOUNTS | AVERAGE INTAKE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fruits juices (pure) | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
| Berry juices (Blueberries, blackberries, raspberries, cranberries, strawberries) (1 cup) | | | | | | | | | |
| Orange juice (1 cup) | | | | | | | | | |
| Apple juice (1 cup) | | | | | | | | | |
| Grape juice (1 cup) | | | | | | | | | |
| Grapefruit juice (1 cup) | | | | | | | | | |
| Pear juice (1 cup) | | | | | | | | | |
| Lemon juice (1 cup) | | | | | | | | | |

| FOODS AND AMOUNTS | AVERAGE INTAKE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Vegetables | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
| Aubergine (raw) (1 cup) | | | | | | | | | |
| Red Cabbage (1 cup) | | | | | | | | | |
| Artichoke (1 cup) | | | | | | | | | |
| Broccoli (raw) (1 cup) | | | | | | | | | |
| Sweet potatoes (1 medium) | | | | | | | | | |
| Rhubarb (1/2 cup) | | | | | | | | | |
| Asparagus (1/2 cup) (cooked) | | | | | | | | | |
| Brussel sprouts (1/2 cup) (cooked) | | | | | | | | | |

| Vegetables *(continued)* | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
|---|---|---|---|---|---|---|---|---|---|
| Celery hearts (1 heart) | | | | | | | | | |
| Soybeans (raw) (1/2 cup) | | | | | | | | | |
| Soy meat products (1/2 cup) | | | | | | | | | |

| Foods (amount) | AVERAGE INTAKE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Drinks | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
| Black tea (1 cup) | | | | | | | | | |
| Green tea (1 cup) | | | | | | | | | |
| Coffee (instant or grounded) (1 cup) | | | | | | | | | |
| Cocoa hot chocolate (1 cup) | | | | | | | | | |
| Red wine (1 glass) | | | | | | | | | |
| Soya milk (1 cup) | | | | | | | | | |

| Foods AND AMOUNTS | AVERAGE INTAKE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Other | Never or less than once/month | 1-3 per month | Once a week | 2-4 per week | 5-6 per week | Once a day | 2-3 per day | 4-5 per day | >6 per day |
| Dark chocolate (1 bar=40g) | | | | | | | | | |
| Milk chocolate (1 bar=40g) | | | | | | | | | |
| Hazelnut, Chestnut (1/2 cup) | | | | | | | | | |
| Apple sauce (1Tbsp) | | | | | | | | | |
| Broadbeans (1 cup) | | | | | | | | | |

**Appendix 10: Physical activity questionnaire for study II**

PHYSICAL ACTIVITY QUESTIONNAIRE

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Did you exercise during the past 6 weeks? ☐ Yes ☐ No

Types of exercise:

……

……

……

-------hour(s) --------day

-------week

------month

**Appendix 11: Acceptability questionnaire for Study II**

ACCEPTABILITY QUESTIONNAIRE

1. How did you find consuming dark chocolate for 12 weeks:
   a. Very acceptable
   b. Somewhat acceptable
   c. Unacceptable

Reasons for unacceptability:

……

……

……

2. Did you notice any change in appetite during this period? Please explain

……

……

……

3. Are you willing to continue having dark chocolate frequently?

Yes ☐ No ☐

Comments:

……

……

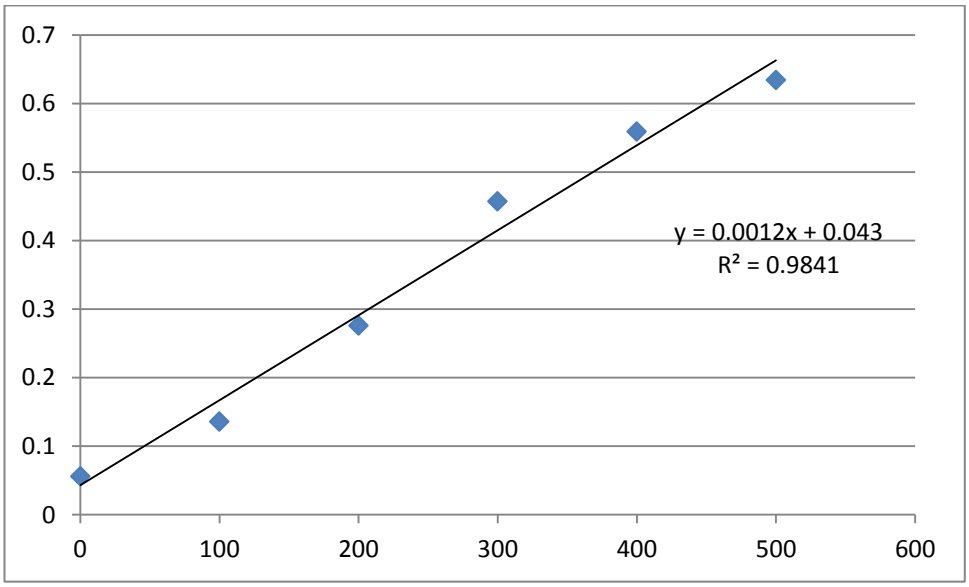
……

**Appendix 12: Standard curves for Folin-Ciocalteu and FRAP methods**

12.1 Folin-Ciocalteu method

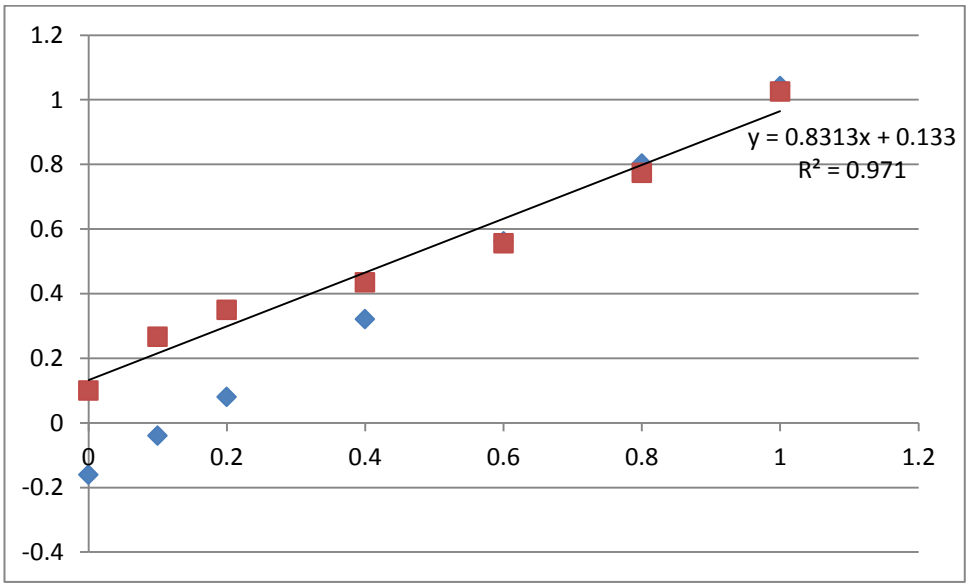
The standard curve for the quantification of total polyphenol content (via Folin-Ciocalteau method) and total antioxidant capacity (via FRAP method) are presented in Figure 12.1 and 12.2, respectively.

12.1: Standard curve for the determination of total phenolics in the urine




Y represents the absorbance in nm and X is the concentration of gallic acid expressed in mg of GAE/day

12.2: Standard curve for the determination of total antioxidant capacity in the urine




Y represents the absorbance in nm and X is the concentration of $Fe^{2+}$ expressed in mM of $Fe^{2+}$/day